Abnormal Response of Osteoblasts to Melatonin in Adolescent Idiopathic Scoliosis

by

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Abstract

Introduction: Adolescent idiopathic scoliosis (AIS) is a complex three-dimensional structural deformity of the spine occurring in adolescence with unknown etiopathogenesis. The reported prevalence rate is about 4% worldwide. Untreated curves could progress and cause significant cosmetic problems and in severe cases morbidities and complications such as cardiopulmonary failures. Current treatments for scoliosis include observation, bracing and corrective surgery with instructments. However, these treatments remain unsatisfactory with the likelihood of curve progression with bracing and the associated surgical complications. Thus, the understanding on the etiopathogenesis of AIS remains the key for the best remedy. Further characterization of the AIS patients in addition to the spinal curvature is important in further delineating the etiopathogenesis of AIS.

One of the well-studied phenotypes of the AIS is the presence of low bone mass. Previous studies have reported low bone mass in about 30% of the AIS patients, and longitudinal follow-up studies also showed that among them more than 80% would have persistent low bone mass after skeletal maturity. From the animal studies, it was shown that pinealectomy in avians and rodents could lead to the development of scoliosis-like deformity. Some studies have reported the beneficial effect of exogenous administered melatonin in the prevention of scoliosis development in these pinealectomized models. Genetic association study of the melatonin receptor 1B gene polymorphism has shown that melatonin receptor 1B polymorphism is associated with the occurrence of AIS. Other studies have suggested the presence of melatonin signaling pathway dysfunction in the osteoblasts of AIS patients. To

explain for the above mentioned observations, it is logical to postulate that there could be abnormality in the response of osteoblasts toward melatonin in AIS patients. With the limited information on the cellular effect of melatonin toward AIS patients, the aim of the current series of study were to evaluate the effect of melatonin on osteoblasts from AIS patients in term of cell proliferation and cell differentiation. Based on the response of the osteoblasts from AIS subjects, AIS subjects were further subgrouped for the analysis and correlation with different anthropometric measurement and bone mineral status. Results were compared with normal controls.

Methods: First part of the study included clinical assessments of 224 severe AIS girls and 120 age and sex matched normal controls. Assessments included anthropometric measurements: body weight, height, arm span, body mass index (BMI). Bone mineral density (BMD) of both bilateral femoral necks and midshafts of non-dominant radius was also assessed by DXA and pQCT, respectively. Second part of the study utilized primary osteoblasts isolated from intraoperative bone biopsies from 13 AIS girls undergoing corrective spinal surgery and 9 control subjects undergoing unrelated trauma surgery. The osteoblasts were cultured *in vitro* and assessed for the effect of melatonin on cell proliferation and differentiation by MTT cell viability assay and alkaline phosphatase activity (ALPase), respectively. In addition, the expression of melatonin receptors, MT1 and MT2, on osteoblasts was examined by western blot. After the characterization of the cellular response of the osteoblasts to melatonin, the subjects were divided into two subgroups according to their response to melatonin in proliferation assay. Detail correlation of the cellular response pattern with the anthropometric cross-sectional parameter and bone mineral

status were then analyzed and results were then compared between groups and the normal controls.

Results: After adjusting for age, the severe AIS girls showed significantly longer arm span and lower body weight, BMI and BMD when compared with healthy female controls. The *in vitro* assays of proliferation on osteoblasts from AIS patients (n=13) demonstrated two different types of responses to melatonin. The first group (Group A) (n=3) showed promoting effect, similar to normal controls, on cell proliferation with physiological dosage (10nM) of melatonin, while the second group (Group B) (n=10) showed inhibitory effect with 10nM of melatonin. Normal controls and group A subjects were also found to have melatonin enhanced osteoblast differentiation and increased ALPase activity while those in Group B showed an inhibitory effect. In semi-quantification of the two types melatonin receptors (MT1 and MT2), the osteoblasts samples showed the absence of MT1 and MT2 receptors inin 4 out of 10 of the osteoblast samples in Group B. These patients had the longest arm span and lowest BMD. Among the Group B subjects, those with the absence of MT2 receptors were found to have an even longer arm span and higher prevalence of family history when compared with the other groups of AIS patients.

Discussion: This is the first report on the abnormal cellular response of osteoblasts in AIS to melatonin. The abnormal responses of osteoblasts may affect the phenotypic expression of AIS patients such as abnormal anthropometric measurement and bone mineral status. In addition to the suggested abnormality in the melatonin signaling pathway in the literatures, the expression of MT2 receptor could contribute significantly to the abnormal response of osteoblasts in AIS. The

observation of the present study points to the speculation that it is likely the AIS subjects consist of a heterogeneous groups of individuals with multifactorial etiopathogenesis. The findings of the current series of studies also provide important evidences on the role of melatonin in the etiopathogenesis of AIS.

摘要

引言：青少年特發性脊柱側凸(Adolescent idiopathic scoliosis , AIS)是複雜的脊柱三維畸形並伴有椎體旋轉，常見於青少年，目前病因未明。青少年中的罹患率約爲 4%。未經治療的側凸可能逐漸加重並導致明顯的外觀畸形，嚴重的側凸尚可導致其他併發症如心肺功能異常。目前對 AIS 的治療方法包括觀察、支具和手術內固定治療。然而這些治療仍然存在一定缺陷，如支具治療失敗、手術的併發症等。因此明確 AIS 的發病機制仍然是獲得最佳治療的關鍵，而對 AIS 患者及其側凸特徵進行研究是對於明確其發病機制至關重要。

AIS 患者研究較多的表型之一是存在低骨量。既往研究表明 30%的 AIS 患者存在低骨量，之後的隨訪研究發現這些患者中 80%患者的低骨量在骨骼發育成熟後仍然存在。動物實驗表明在禽鳥類和齧齒類動物通過切除松果體可以建立類似特發性的脊柱側凸模型，並且有部分研究顯示通過外源性補充褪黑素可以阻止松果體切除的動物發生脊柱側凸。基因相關性分析研究顯示褪黑素 1B 受體的基因多態性與 AIS 的發生相關。其他研究還顯示 AIS 患者成骨細胞中褪黑素信號通路存在異常。這些均提示 AIS 患者中褪黑素對成骨細胞的作用可能存在異常。目前尚缺乏在細胞水準對 AIS 患者褪黑素功能的研究報導，因此本課題對褪黑素在 AIS 患者成骨細胞的增殖和分化中的作用進行研究，並根據成骨細胞對褪黑素的反應將 AIS 患者分組以進一步分析其人體測量學參數及骨密度情況並與正常對照比較。

方法：第一部分實驗選擇 224 例重度脊柱側凸患者及 120 例年齡、性別匹配的正常對照，對其進行人體測量學分析，包括體重、身高、臂長並計算人體品質指數(body mass index, BMI)，並採用雙能 X 線測定雙側股骨頸的骨密度(Bone mineral density, BMD)及 pQCT 測量非優勢側橈骨中段的骨強度。第二部分實驗從 13 例行手術治療的 AIS 和 9 例創傷手術的正常對照獲取骨組織並在體外原代培養成骨細胞。褪黑素對成骨細胞的增殖和分化的作用分別通過測定褪黑素刺激後的細胞數量和鹼性磷酸酶活性來評價。成骨細胞中褪黑素受體(MT1 和 MT2)的表達採用 Western Blot 檢測。根據褪黑素對 AIS 患者成骨細胞增殖的影響將患者分組，分析各組細胞對褪黑素的反應並與人體測量學參數和骨礦物情況作相關分析，並比較各組間和正常對照的結果。

結果：經過年齡校正後和正常健康女孩對照比較，重度 AIS 患者臂長較長、體重較低、BMI 和 BMD 均較低。褪黑素對不同 AIS 患者成骨細胞的增殖顯示出兩種作用，據此將 AIS 患者分爲兩組。生理學劑量的褪黑素(10nM)對成骨細胞增殖有促進作用的爲 A 組，有抑制作用的則爲 B 組。結果顯示在 A 組成骨細胞中褪黑素對其鹼性磷酸酶活性有促進作用，而在 B 組則爲抑制作用。半定量檢測的結果顯示所有成骨細胞均表達 MT1 受體，但 B 組中有 4 例成骨細胞沒有檢測到 MT2 受體的表達，但其他 AIS 及正常對照的成骨細胞中均有表達。與 AIS 的其他表型作相關分析發現，B 組患者的臂長較 A 組長，BMD 較 A 組低。B 組中未檢測到 MT2 受體表達的患者較其他組患者有較長的臂長和較高的脊柱側凸家族史幾率。

討論：本研究首次報導了 AIS 患者成骨細胞在細胞水準對褪黑素的異常反應。成骨細胞的異常反應可能影響到 AIS 的一些表型，如異常的人體測量學參數和骨質礦物情況。除了報導的褪黑素信號通路異常外，MT2 受體表達異常也可能影響到褪黑素對 AIS 成骨細胞作用。本研究結果提示 AIS 患者可能存在不同的發病機制，AIS 患者可能存在不同的病因。本研究的發現爲研究褪黑素在 AIS 發病機制中的作用提供了重要證據。

Abbreviations

α-MEM	Alpha-minimal essential medium
4-P-PDOT	4-phenyl-2-propionamidotetralin
AA-NAT	Arylalkylamine-*N*-acetyltransferase
ACL	Anterior cruciate ligaments
AIS	Adolescent idiopathic scoliosis
ALPase	Alkaline phosphatase
ANOVA	One-way analysis of variance
APS	Ammonium persulfate
BA	Bone area
bALP	Bone alkaline phosphatase
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BMC	Bone mineral contents
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenetic protein
BMU	Basic multicellular unit
BO line	Basionopisthion reference line
CBFA-1	Core binding factor-1
CO_2	Carbon dioxide
CSA	Cross-sectional area
CT	Computed tomography
Cyclic AMP	Cyclic adenosine monophosphate
DEA	Diethanolamine

Dlx5	Distalless homeobox 5
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DXA	Dual energy x-ray absorptiometry
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FGFs	Fibroblast growth factors
GH	Growth hormone
Gpp(NH)p	5'-Guanylimidodiphosphate
GTP	Guanosine-5'-triphosphate
HIOMT	Hydroxyindole-*O*- methyltransferase
IGF-1	Insulin-like growth factor 1
IGF-1R	IGF-1 receptor
Ihh	Indian hedgehog
IL-6	Interleukin-6
JAK	Janus kinase
MAPK	Mitogen activated protein kinase
Melatonin	N-acetyl-5-methoxytryptamine
MGP	Matrix GLA protein
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cells
MTNR1B	Melatonin 1B receptor
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT	Nitro blue tetrazolium
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PINX	Pinealectomy
Pl3K	Phosphatidylinositol 3-kinase
PNPP	p-Nitrophenyl phosphate
PNS	Penicillin/neomycin/streptomycin antibiotic cocktail
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
PTHrP	PTH-related protein
QLPSD	Quality of life profile for spine deformities
RANKL	Receptor activator of NF-kappaB ligand
RIPA	Radioimmunoprecipitation assay
RFLP	Restriction fragment length polymorphism
RT-PCR	Real-time polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS–PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
SPA	Single energy photon absorptiometry
SSEP	Somatosensory-evoked potentials
STAT	Signal transducers and activator of transcription
TEMED	Tetramethylethylenediamine
TBST	Tris-buffered saline with Tween-20
TLSO	Thoracolumbosacral orthosis
TWIST	Twist homolog

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Major Conference Presentations

1. **Man GCW**, Yeung HY, Wang WJ, Lee KM, Ng BKW, Hung WY, Ng TB, Qiu Y, Cheng JCY. A Study of the Effect of Melatonin on the Proliferation & Differentiation of Osteoblasts in Adolescent Idiopathic Scoliosis, Scoliosis Research Society 43rd Annual Meeting, September 10-13, 2008, Utah, USA
   (Awarded John Moe's Award for best basic science poster)
   [Refer to Appendix I]

2. **Man GCW**, Yeung HY, Wang WJ, Lee KM, Ng BKW, Hung WY, Ng TB, Qiu Y, Cheng JCY. A Study of the Effect of Melatonin on the Proliferation & Differentiation of Osteoblasts in Adolescent Idiopathic Scoliosis, The 15th International Meeting on Advanced Spine Techniques, July 8-11th , 2008, Hong Kong, SAR

3. **Man GCW**, Wang WJ, Yeung HY, Ng BKW, Lee KM, Hung WY, Qiu Y, Cheng JCY. **A** Study of the Effect of Melatonin on the Proliferation and Differentiation of Osteoblasts in Adolescent Idiopathic Scoliosis, the 7th International Research Society of Spinal Deformities, July 9-12th 2008, Liverpool, UK. Pp

4. **Man GCW**, Yeung HY, Wang WJ, Lee KM, Ng BKW, Hung WY, Ng TB, Qiu Y, Cheng JCY. A Study of the Effect of Melatonin on the Proliferation and Differentiation of Osteoblasts in Adolescent Idiopathic Scoliosis, The International Congress of Osteoporosis and Bone Research 2008, October 22-25, 2008, Beijing, China.

5. **Man GCW**, Yeung HY, Wang WJ, Lee KM, Ng BKW, Hung WY, Ng TB, Qiu Y, Cheng JCY. A Study of the Effect of Melatonin on the Proliferation and Differentiation of Osteoblasts in Adolescent Idiopathic Scoliosis, the Chinese Orthopedics Association 3rd Annual Congress, November 12-16, 2008, Suzhou, China.

6. **Man GCW**, Yeung HY, Wang WJ, Lee KM, Ng BKW, Hung WY, Ng TB, Qiu Y, Cheng JCY. The Abnormal Effect of Melatonin on the Proliferation & Differentiation of Osteoblasts in Adolescent Idiopathic Scoliosis. The 28th Annual Congress of Hong Kong Orthopedics Association, November 29-30th 2008, Hong Kong, SAR

7. **Man GCW**, Wang WJ, Yeung HY, Lee KM, Ng BKW, Hung WY, Ng TB, Qiu Y, Cheng JCY. A Study of the Effect of Melatonin toward the Proliferation and Differentiation of Osteoblasts in Adolescent Idiopathic Scoliosis, the 55th Annual Meeting of the Orthopedics Research Society, February 22-29, 2009, Las Vegas, Nevada, USA.

Publications in Preparation

1. The effect of melatonin on the proliferation and differentiation of osteoblasts in adolescent idiopathic scoliosis Target Journal: Spine (Manuscript In preparation)

2. Melatonin receptor expression of osteoblasts in adolescent idiopathic scoliosis Target Journal: J Pineal Res (Manuscript in preparation)

Study Flowchart

Background

- Adolescent idiopathic scoliosis is a complex three-dimensional structural deformity of the spine occurring most commonly in adolescence with a prevalence of 4%.
- The current treatments, including observation, bracing and surgical correction, are not totally satisfactory and are associated with different morbidity and complications. The ultimate remedy for progressive AIS patients requires the proper understanding of the etiopathogenesis and thus the specific and appropriate treatment regime.
- Genetic factors, abnormal skeletal growth, metabolic dysfunction, neurosensory disturbance, and biomechanical stress are the main groups that have been postulated to be associated with the etiopathogenesis of AIS.
- Experimental animal model of pinealectomized chicken and melatonin-deficient mice were found to develop similar spinal deformity.

Previous Studies

- Melatonin enhances BMD in ovarectomized rats.
- Melatonin prevents scoliosis development in pinealectomized rodents and avians.
- Dose-dependent concentrations of melatonin promote human osteoblasts to proliferation, differentiation and mineralization.

- Clear evidences of the importance of melatonin on bone remodeling.
- Existence of melatonin signaling pathway dysfunction in osteoblasts isolated from severe AIS patients.
- Melatonin 1B gene polymorphism associated with the occurrence of AIS.
- **Hypothesis:**

  Abnormal response of osteoblasts to melatonin is present in girls with AIS.

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Objectives

1. To study the anthropometric parameter and BMD of girls with severe AIS, in comparison with healthy female adolescent controls.
2. To determine the effect of melatonin on proliferation and differentiation of osteoblasts isolated from intraoperative bone biopsies of AIS patients.
3. To detect the expression of melatonin receptors on osteoblasts isolated from intraoperative bone biopsies of AIS patients in comaprison with healthy normal controls.

Question 1:

Is there any systemic abnormality in skeletal growth and BMD in girls with severe AIS requiring surgerical treatment?

Methods:

- Cross sectional study
- Anthropometric measurements (Height, Weight, Arm span and Body Mass Index) of severe AIS girls with age-matched healthy girls.

Findings:

- Severe AIS girls were lighter and leaner than the controls.
- Severe AIS girls have a significantly lower BMI than the control.
- Arm span was significantly longer in severe AIS girls than in normal controls

Methods:

- Cross sectional study
- BMD measurements of both femoral neck and midshaft of non-dominant radius.
- Dual-energy x-ray absorptiometry
- Peripheral Quantitative Computerized Tomography
- Severe AIS girls versus Controls.

Findings:

- The BMD of both femoral necks was significantly lower in severe AIS subjects.
- There was no statistical difference on the BMD in the midshaft of distal radius between severe AIS girls with controls.

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Implications:

- There is systemic abnormality in bone growth of severe AIS girls.
- Disproportionally active endochondral ossification in severe AIS girls.
- The change in arm span may help in monitoring the abnormal growth in AIS patients.

Implications:

- The presence of generalized low BMD in severe AIS girls.
- Possible implication on abnormality in bone remodeling of severe AIS girls.

Summary:

- Abnormality of anthropometric measurements and BMD in severe AIS girls.
- Possible abnormality in bone formation in AIS girls.
- Previous reports of melatonin signaling dysfunction in AIS osteoblasts.
- Is there a relationship between the melatonin dysfunction with the abnormal phenotypes observed?

Question 2:

Is there evidence of abnormal melatonin response in osteoblasts from patients with AIS?

Methods:

- Isolated osteoblasts from AIS patients, undergoing corrective surgery, and confirmed by morphology and presence of alkaline phosphatase.
- Tested the effect of melatonin on proliferation of AIS osteoblasts by MTT viability assay.
- Tested the effect of melatonin on differentiation of AIS osteoblasts by alkaline phosphatase activity.

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Findings:

- Abnormal response to melatonin in AIS osteoblasts.
- Two different responses to melatonin occurred in the AIS osteoblasts.
- On proliferation
  - Group A: Melatonin enhanced proliferation.
  - Group B: Melatonin showed a drop in proliferation.
- On differentiation
  - Group A: Melatonin enhanced differentiation.
  - Group B: Melatonin showed a drop in differentiation.

Implications:

- Abnormality in AIS osteoblasts toward melatonin.
- This implies possible heterogeneity in the AIS populations in the response toward melatonin.

Summary:

- Abnormal response to melatonin in osteoblasts isolated from AIS girls.
- Previous reports of melatonin signaling dysfunction in AIS osteoblasts.
- Are melatonin receptors present in osteoblasts from the Group B AIS patients?
- Abnormal response to melatonin in osteoblasts isolated from AIS patients

Question 3:

Whether there is abnormality in the expression of melatonin receptors in AIS osteoblasts?

Methods:

- Isolated osteoblasts from AIS patients undergoing corrective surgery, and confirmed by morphology and presence of alkaline phosphatase.
- Investigated the protein expression of the melatonin receptors in AIS osteoblasts by Western Blot.
- Correlation of the melatonin response to clinical phenotypes.

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Findings:

- All osteoblast samples, both normal controls and AIS subjects, showed positive MT1 receptor expression by Western Blot.
- Normal controls and Group A showed expression of MT2 receptors.
- Group B showed significantly lower expression of MT2 receptor in selected samples of AIS osteoblasts.
- Further subclassification into Group B2 for those with no observable MT2 expression by Western blot.
- By RFLP, all the AIS osteoblasts showed the presence of MT2 gene.
- Group B2 has generalized longer arm span than Group A and Group B1.
- Rate of familial history is higher in Group B2 than Group A and Group B1.

Implications:

- This further implies heterogeneity in the AIS populations.
- In the group B, further classification of a subgroup from the lack of observable MT2 receptor expression by Western blot.
- The result could imply that low or absent MT2 receptor might contribute to the disproportional endochondral ossification, thus resulting in having longer arm span and lower BMI in affected AIS patients.
- Findings support the classifications on the melatonin signaling pathway dysfunction reported by Moreau *et al.*

Conclusions:

- Confirmation of abnormal bone growth in severe AIS girls.
- Severe AIS girls have a lower BMI and longer arm span than normal controls.
- Severe AIS girls demonstrated a generalized low BMD when compared with healthy normal controls.
- Based on the response to melatonin and presence of MT2 receptors, the AIS osteoblasts can be classified into three subgroups (Group A, Group B1 and Group B2).

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- Group A osteoblasts showed enhanced proliferation and differentiation with the addition of physiological and pharmacological concentrations of melatonin.
- Group B1 osteoblasts showed inhibited proliferation and differentiation with the addition of physiological and pharmacological concentrations of melatonin.
- Group B2 osteoblasts showed inhibited proliferation and differentiation to melatonin, with relatively low or absent MT2 receptors.
- Longer arm span and lower BMI observed in Group B2 AIS patients.
- Higher familial history in Group B2 AIS patients.

Further Studies

1. Prospective follow-up study on the anthropometry of severe AIS patients with long arm span.
2. Cyclic AMP assessment to be conducted to study the possible association with the observed abnormal functional response in the AIS osteoblasts
3. The material property and microarchitecture of the bone in AIS girls.
4. Possible bone markers during the growth spurt in AIS girls.
5. Analysis on the structural changes of the melatonin receptors.
6. Sub-classified the AIS patients by the abnormal melatonin response to correlate with the onset and progression of AIS.

Chapter 1 Study Background

1. Introduction

1.1. General Overview of Adolescent Idiopathic Scoliosis (AIS)

Idiopathic scoliosis is the most common consulted pathological deformity of the spine. By definition, it is a complex three-dimensional structural lateral deformity of the spine with vertebral rotation to an otherwise healthy individual (Figure 1.1). It is a lifelong, probably systemic condition of unknown cause. Current clinical screeing test diagnosis of scoliosis is based on the forward bending test. If a hump is noted, the suspected patient would be sent for an x-ray to confirm the diagnosis. The standard method for assessing the curvature is by measuring the Cobb's angle. The classification of scoliosis is divided into 3 groups depending on the time of onset: 1) infantile (age below 3 years old); 2) juvenile (age 5 to 8 years old), and 3) adolescent (age 10 years old to skeletal maturity) (James, 1954). According to radiographic classification of the curvature's apex region, it can be further described as cervical, thoracic, thoracolumbar and lumbar curves. Among the different subtypes of idiopathic scoliosis, adolescent idiopathic scoliosis is the most common form and accounts for 80% of the scoliosis.

The occurrence of AIS, using a cut-off point of 10^{o} Cobb or more, can vary from 2 to 2.5% among different countries and ethnical race (Daruwalla *et al.*, 1985, Kane, 1977, Lonstein, 1988, Robin, 1990, Shohat *et al.*, 1988). In Hong Kong, the prevalence of AIS is about 4% as reported by the Department of Health. The occurrence of scoliosis is similar in males and females with small curves of 10^{o} (Rogala *et al.*, 1978). However, the incidence of the AIS patients with clinical

significant curve that is with Cobb's angle greater than 20° is significantly higher in female than in male (Rogala *et al.*, 1978) This indicates that gender may play a vital role toward progression. Similarly, the female to male ratio requiring treatment is 7:1, with approximately 0.1% warranting the need for corrective surgery (Taylor *et al.*, 2000). Although less than 10% of epidemiological studies truly reported that positively screened scoliosis would require any treatment, those in need of attention would benefit from a better understanding of the etiology of this disorder (Brooks *et al.*, 1975, Bunnell, 1986, Montgomery and Willner, 1997, Rogala *et al.*, 1978, Weinstein and Ponseti, 1983).

1.2. Natural History

The natural history of AIS plays an important role in helping the clinical management and research aiming at understanding the etiopathogenesis (Asher and Burton, 2006, Weinstein *et al.*, 2003). Upon skeletal maturity, minor loading with a large curve would inevitably be more difficult to prognosticate (Van Goethem *et al.*, 2007). Previously, Weinstein *et al.* (1983) reported that 68% of the 133 curves in 102 patients progressed after skeletal maturity. It was found that curves initially less than 30° Cobb generally would not progress and single thoracic curves, between 50° to 75° Cobb, are more likely to progress (Ascani *et al.*, 1986, Weinstein and Ponseti, 1983). In addition, large apical vertebral rotations in single thoracic curves have been shown to contribute to curve progression (Weinstein, 1999). For double curves, the position of the major curve (lumbar > thoracic) and direction right lumbar > left lumbar) could affect the curve progression (Weinstein and Ponseti, 1983).

In a 50-year follow up study on untreated scoliosis (Weinstein *et al.*, 2003), Weinstein *et al.* (2008) demonstrated little physical impairment, other than back pain and cosmetic concerns. Likewise, the mortality rate of patients suffering from scoliosis is more or less similar to the average adolescents in the population. Significant differences only arise from patients carrying a thoracic curve greater than 80° Cobb angle, which could result in progressive pulmonary failure. Typical discomforts reported from scoliotic patients include back pain (Mayo *et al.*, 1994), pulmonary symptoms (Pehrsson *et al.*, 1992, Weinstein *et al.*, 1981), low self-esteem (Weinstein *et al.*, 2003), and low bone mineral density (BMD) (Burner *et al.*, 1982, Cheng and Guo, 1997).

Based on these findings, AIS would not result in increased mortality rate. However, it is still not clear whether AIS is the cause of death reported from cardiopulmonary failure in some patients. In a 25 years study, Branthwaite *et al.* (1986) reported that 11 out of 800 AIS patients attending a chest clinic died from cardiopulmonary failure. An eleven-year-old AIS patient, diagnosed with 40 Cobb, died from cardiopulmonary failure in adulthood, with a curve progression over 150° (Branthwaite, 1986). However, in another study, respiratory failure only occurred in AIS patients with a predicted low vital capacity (< 45%) and a larger scoliotic angle (> 110°) (Pehrsson *et al.*, 2001, Pehrsson *et al.*, 1992).

Typical pulmonary symptoms in shortness of breath is commonly associated with idiopathic scoliosis (Pehrsson *et al.*, 1992, Weinstein *et al.*, 1981). It is more common in AIS patients carrying a thoracic curve greater than 80° Cobb or with increased vertebral rotation near the thoracic rib cage, though larger double curves

may also have similar association (Weinstein *et al.*, 1981). Other than that, the prevalence of back pain has been reported occasionally in AIS patients (Mayo *et al.*, 1994, Weinstein *et al.*, 2003). Although pain severity is not related to curve size (Mayo *et al.*, 1994), curve pattern seems to increase with pain (Weinstein and Ponseti, 1983). Thoracolumbar curves would generate greater back pain than double curves (Ascani *et al.*, 1986). Another major setback in AIS patients is low self esteem (Weinstein *et al.*, 2003). This is significantly higher in younger female AIS patients with small curves than control (Goldberg *et al.*, 1994). Likewise, other health impairments, including hypertension (Pehrsson *et al.*, 1992) and neurological impairment (Jackson *et al.*, 1983), are seldom reported.

1.3. Current Treatments

Current medical treatment for AIS depends on the nature and severity of the spinal curve. There are a number of criteria affecting the choice of treatments. The criteria include the age of the patient, skeletal maturity, curve types, level of curvature and progression status. The main objective of the treatment is to control the curve from progression and to minimize the need for surgery with improvement on cosmetic appearance. The treatment is divided into 2 categories: non-operative and operative treatments.

1.3.1. Non-operative Treatments

Non-operative treatment of scoliosis includes observation and bracing. Observation

can only be employed for skeletal immature patients with a Cobb's angle less than 20 degree. Upon reaching skeletal maturation, any curvature less than 40 can still be treated by monitoring alone (Tang *et al.*, 2003). Other than that, bracing would be recommended by the clinicians (Figure 1.2). Patients are advised to wear braces for twenty-two to twenty-four hours a day. The most typical braces used are the Milwaukee (Noonan *et al.*, 1997), Boston (Bunge *et al.*, 2008, Morton *et al.*, 2008) , Charleston bending (Gepstein *et al.*, 2002, Price *et al.*, 1990), and thoracolumbosacral orthosis (TLSO) (Fernandez-Feliberti *et al.*, 1995, Kahanovitz *et al.*, 1984). The Milwaukee brace is for scoliosis patients with apex higher than T8. This brace consists of metal rods attached to pads at the hips, rib cage and neck. The Charleston bending brace is used at night for flexibility to bend the spine in opposite directions. Another type of brace is the underarm TLSO brace, which uses rigid plastic to surround the lower rib cage, abdomen and hips for apex under T8. Climent and Sanchez (1999) conducted a cross-sectional study to determine the type of bracing on self-perceived health status. One hundred and two skeletally immature patients with spinal deformities (75% with scoliosis) were subjected to different braces (Milwaukee, Boston, Charleston bending and TLSO braces) for routine biannual follow-up. The degree on health status was assessed by a specific instrument on the Quality of Life Profile for Spine Deformities (QLPSD). Milwaukee brace-treated patients showed a significantly higher impairment in the quality of life than patients treated with the other 3 braces, in terms of psychosocial functioning and back flexibility. In the event of different orthoses with proven similar effectiveness in controlling the curvatures, bracing with the lowest impact on the quality of life should be recommended. However, controversies still exist with respect to the effectiveness of bracing, especially in the different types of bracing,

bracing duration, type of curve, severity of curve and long term outcome.

1.3.2. Operative Treatments

Surgical correction was first initiated in 1914 (Hibbs, 1911). It was used as the last resort in spinal curve correction. Before proceeding with corrective surgery, many factors in the patient's clinical profile have to be considered. These include the curve magnitude, skeletal age, curve characteristic, cosmetic appearance and potential risk of curve concerned. Evidently, patient preference is one of the important consideration on whether surgery would be performed (Greiner, 2002). In general, patients having a thoracic curve with an angle of over 45° Cobb and a high potential of progression (Clark, 2008) during adolescence would be advised to undergo corrective surgery. Other curve patterns would generate more problems due to the risk of lower back pathology and pain into the low lumbar region of the spine (Weinstein *et al.*, 1981). The main purpose of corrective surgery is to prevent further progression and minimize curve angle, restoration of trunk balance and improvement on cosmetic appearance. Pehrsson *et al.* (2001) showed that there was improvement in the pulmonary function of surgically-operated AIS patients. In the 25-year follow-up, the vital capacity of the pre-operated and operated AIS patients increased from 67% to 84%, respectively. The two main principles of surgery are: 1) using instrumentation to correct the spinal curvature and improvement of balance and 2) maintaining the solid bone fusion at the corrected position to prevent further deterioration. Corrective surgery can be achieved through posterior, anterior or combined approaches. The type of surgery performed would depend on the magnitude and stiffness of the operated curve. With documented solid fusion of

the spine usually within six months to one year postoperatively, the patient can then resume an active normal lifestyle (Bridwell *et al.*, 1999).

1.3.3. Complications of Treatments

In non-operative treatments, monitoring alone can lead to bias among different clinicians. Different medical practices and measurements can cause misinterpretation of the actual curve angle (Theologis *et al.*, 1997). With regards to bracing, some centers have different implications on significantly controlling curve progression (Karol, 2001, Milbrandt and Johnston, 2005, Sponseller *et al.*, 2000). Although controversies still exist in reducing the need for surgery, promising results have been reported in some centers (Rigo, 2003, Weiss, 2003). Results showed a reduction in the frequency of surgery to 50%. Similarly, the use of bracing would not be effective for a curve greater than 45 degrees and the presence of significant thoracic lordosis. Likewise, the stiffness of the bracing might cause discomfort and fatigue in some patients (Gabos *et al.*, 2004, Mac-Thiong *et al.*, 2004, Roach, 1999).

Spinal corrective surgery with instrumentation is a major surgery and can be associated with significant morbidity. As a safeguard, a thorough pre-operative assessment of blood clotting, radiological curve location, somatosensory evoked-potentials and cardiopulmonary function would usually be conducted (Hausmann *et al.*, 2003, Lauder, 2007, Winkler *et al.*, 1998). After the surgery, postoperative care and recovery would be needed. Most importantly, corrective surgery can only correct the cosmetic appearance of the curve. However, health-related signs and symptoms of AIS cannot be corrected (Weiss *et al.*, 2008).

There are reports of curve reoccurrence and progression in the young patients until skeletal maturity is reached (Kesling *et al.*, 2003, Lee and Nachemson, 1997). This is known as 'crankshaft phenomena'. Hence, the best way to overcome the problem of bracing and surgery would be to find an alternate way to treat scoliosis. In order to do so, it would be most essential to have a better understanding of the etiology and etiopathogenesis of adolescent idiopathic scoliosis.

1.4. Additional Phenotypes Abnormalities

1.4.1. Growth and Development

Generally, it is recognized that the curve onset and progression in AIS are closely related to growth. Many studies have analyzed the anthropometry of both normal and AIS girls (Burwell *et al.*, 2006, Goldberg *et al.*, 2006, Shohat *et al.*, 1988, Siu King Cheung *et al.*, 2003, Starcevic-Klasan *et al.*, 2008, Ylikoski, 2003). Most of these studies showed that AIS patients were taller and more slender than normal adolescents (Burwell *et al.*, 2006, Goldberg *et al.*, 2006, Siu King Cheung *et al.*, 2003), while some showed no significant difference (Skogland and Miller, 1981, Ylikoski, 2003). In a cohort study with 11 years follow up, Nissinen *et al.* (2000) showed that body height, sitting height and growth of sitting height were higher in pre-scoliotic children, however, this was insignificant toward development of AIS. Similarly, Cheung *at al.* (2006) demonstrated that scoliotic girls were significantly taller and had longer limbs than normal control after the onset of puberty.

In order to compare growth between normal and AIS individuals, Zorab *et al.* (1971)

measured the total urinary hydroxyl proline profile excreted from normal and AIS children. The increase in urinary concentration would indicate a high turnover of bone collagen, corresponding with bone growth and repair. The results demonstrated a higher excretory activity in AIS patients over control, which indicate either an increase in bone growth or bone remodeling in AIS patients. However, Clark *et al.* (1980) measured the same parameters in 21 AIS patients and found no difference between the normal control and AIS patients. Also, fluctuation in growth hormone has been mentioned to be a causative factor in the development of AIS. Controversial findings have been established from different centers of investigations. Willner *et al.* (1976) found a higher basal growth hormone and somatomedin-A in serum samples of AIS patients compared with controls. However, other authors failed to demonstrate any substantial difference in these two indicators in AIS patients and healthy controls (Misol *et al.*, 1971, Spencer and Zorab, 1977).

Although much of the findings seemed conflicting among different centers, it is a fact that AIS patients are generally taller and more slender than normal controls. This could indicate a faster growth pattern in AIS patients, whose spine is longer and slimmer, and more prone to the development of scoliosis.

1.4.2. Delay Sexual Maturation

Characterization of normal puberty in adolescents can be indicated by chronological age, height and weight variation, skeletal growth and sexual maturation (Sanders *et al.*, 2007). Idiopathic scoliosis progression in adolescence has been closely related to patient maturity status (Burwell, 2003, Goldberg *et al.*, 1993). The most

commonly used indicators for assessing maturity in scoliosis include Risser signs, chronological age and the age at menarche. Although the stage of ossification of the iliac apophysis, or Risser sign, is most commonly used for determining skeletal maturation in AIS patients, it has been shown to be poorly correlated with the curve progression phase (Izumi, 1995). Thus, in choosing the type of indicator, it should be easily detected by orthopedic surgeons and correlate significantly with curve progression.

In 1993, Goldberg *et al.* (1993) conducted a study to compare the pubertal growth spurt of Irish AIS girls. The age of menarche in AIS girls was shifted significantly 0.39 years earlier than the national mean for Irish girls. In terms of addressing progression, it was found that the group with early menarche tends to have a stable curvature. However, those with a late onset of menarche, at the age of 13.83 years, tended to progress. Similarly, other findings also showed similar interpretations of early menarche in AIS girls (Ahl *et al.*, 1988). Although there was a significant difference in that AIS patients had a taller height at the time of diagnosis, the difference between the AIS patients and control disappeared upon maturation. On the contrary, Warren *et al.* (1986) found that ballet dancers with scoliosis had a late onset of menarche and were taller than healthy controls. However, this finding was criticized the ground that on ballet dancers represented only a minority of the scoliotic population (Goldberg *et al.*, 1993). Likewise, it is likely that ballet dancers would escape from the early screening test to prevent bracing, as it would end their dance life. Hence, ballet dancers with AIS would likely be screened with a late menarche. Implications of an early onset of menarche matched with the clinical observations that AIS girls are taller and slender, yet stronger evidences are

still needed for this being an absolute growth disturbance.

1.4.3. Osteopenia

Association of osteopenia with AIS was first reported by Wynne-Davies (1968) in UK from a family survey. Similar observations were reported by different authors (Burner *et al.*, 1982, Cheng and Guo, 1997, Cook *et al.*, 1987a, Thomas *et al.*, 1992, Wynne-Davies, 1968). Low bone mineral density was reported in AIS patients from different part of the world (Carter and Haynes, 1987, Cheng and Guo, 1997, Matzen *et al.*, 1984). However, there is no evidence on association with regional or ethnical specificity. Current clinical evaluations on BMD include single-energy photon absorptiometry (SPA) (Runge *et al.*, 1980, Weinstein *et al.*, 1991), quantitative computed tomography (CT) (Cheng *et al.*, 2006, Yeung *et al.*, 2006) and dual-energy x-ray absorptiometry (DXA) (Cheng and Guo, 1997, Hung *et al.*, 2005).

Cheng *et al.* (1997, , 1999a) have clearly demonstrated the presence of low BMD in female AIS patients. In the cross-sectional study, assessment was conducted on the lumbar spine and proximal femoral BMD with DXA in 81 AIS girls (Cheng *et al.*, 1999a). In all the measured regions, the AIS patients were found to have a lower BMD between 6.9 - 30.4%. In addition, Cheng *et al.* (1999a) found that in 33.3% and 20.1% of the 71 AIS patients, the proximal femoral BMD was 1 SD and 2 SD below age- and sex-matched normal healthy controls, respectively.

Past indications have shown the reduced BMD of premenopausal female adults with a past history of AIS (Cheng and Guo, 1997, Cook *et al.*, 1987c). This suggested

that osteopenia might be a persistent problem in AIS patients. Cheng *et al.* (1999a) conducted a follow-up study of the BMD of 14 AIS patients of more than 2 SD below the mean normal value. Assessment was carried out by DXA with follow-up as long as 3 years longitudinally. Comparing the BMD of the AIS patients at initial and follow-up evaluation, the z-score decreased from -2.96 to -3.84. This indicated a significant difference in the rate of increase of the femoral neck BMD (1:2) between AIS patients and age-matched controls. There is a chance that the lower BMD in AIS would persist and manifest possibly into osteoporosis in adulthood. Cheng *et al.* (1997) and Cook *et al.* (1987b) reported no difference in the proximal femoral BMD of the two hips between AIS patients and normal healthy controls. This disproved the lower femoral BMD in AIS being secondary to the spinal deformity and the asymmetrical mechanical loading of the hips and spine. Likewise, a cross sectional, case control study failed to indicate bracing during growth could affect the BMD (Snyder *et al.*, 2005, Snyder *et al.*, 1995). In addition, Hung *et al.* (2005) conducted a prospective study on 324 AIS female patients with BMD profile of the spine and both hips. Longitudinal measurements on BMD were conducted on these patients until they reached skeletal maturity or curve progressed greater than 6^{o}. By using complicated statistics, the prevalence of curve progression could be predicted. This demonstrated that the measurement of BMD, at the time of diagnosis, may serve as an additional objective measurement in predicting curve progression in AIS.

Intrinsic factors have been known to affect skeletal development and BMD in AIS (Krall and Dawson-Hughes, 1993). Sexual maturation has shown a significant relationship between early menarche and higher peak bone mass (Slemenda *et al.*,

1994). There is speculation that the mechanically weakened spinal column in the potential osteopenic patients may be inclined to scoliosis development during the rapid growing peri-pubertal period. There is strong evidence that intraskeletal mechanisms may participate in the pathogenesis of AIS (Cheng *et al.*, 1999a). Further studies on the regulation of endochondral and membranous bone formation would be needed to elucidate the etiological role of osteopenia in AIS.

1.5. Bone Modeling and Remodeling in Adolescents

The human endoskeleton is made up of rigid bone tissues, which is comprised of cortical and trabecular bone. The cortical bone, or compact bone, is a rather dense tissue with mass penetration by blood vessels through a network of canaliculi. These can be found in the shaft of long bones. While the trabecular bone, or cancellous bone, is porous and located near joint surfaces at the end of long bones and within the vertebrae. To ensure a healthy metabolism on bone quality, old bone from the skeleton would be removed (bone resorption) with new bone being generated (bone formation). This process is known as bone remodeling. These processes also control the reshaping or replacement of bone during growth and following injuries. Imbalance in the regulation on the bone modeling and remodelingcan result in different metabolic bone disease such as osteoporosis.

1.6. Bone Development

1.6.1. Endochondral Ossification

Endochondral ossification, or intracartilaginous ossification, could be defined as one of the two processes during fetal development of the mammalian skeletal system in which bone tissue is created. This process is necessary for the rudimentary formation of long bones (Forriol and Shapiro, 2005), growth in length of long bones (Brighton *et al.*, 1973) and healing of bone fractures (Brighton and Hunt, 1986). Vertebral growth can also be accounted for by endochondral ossification at the two epiphysial growth plates on the upper and lower surfaces of the vertebral body (Knutsson, 1966, Roaf, 1960). The primary site of ossification begins at the middle of diaphysis (shaft) (Martin *et al.*, 1998). Under vascularization, the perichondrium becomes the periosteum, which would later differentiate into osteoblasts. During this process, there is a rich secretion of collagen and other proteoglycans found around the site of ossification. The formed osteoblasts would then secrete osteoid against the shaft of the cartilage to become a support for the new bone. Hypertrophic chondrocytes would begin to migrate toward the midsection of the ossification center, with secretion of alkaline phosphatase. Likewise, the hypertrophic chondrocytes would secrete VEGF to induce the sprouting of blood vessels in the perichondrium. The newly formed blood vessel would carry the hemopoietic cells (which further differentiate into bone marrow cells), osteoprogenitor cells (which further differentiate into osteoblasts cells) and other cells into the cavity. With the occurrence of apoptosis to the hypertrophic chondrocytes, the calcification of the matrix occurred to create a cavity within the bones (Sawae *et al.*, 2003). The osteoprogenitor-differentiated osteoblasts would

use the calcified matrix as a scaffold to secrete osteoid, which is composed mainly of Type 1 collagen, to form the bone trabecula. At the end, osteoblasts would break down the spongy bone to form the medullary cavity. During puberty, various regulators would interact in endochondral ossification toward growth. These factors include growth hormone (GH), insulin-like growth factor 1 (IGF-1), parathyroid hormone (PTH) and PTH-related protein (PTHrP) (Van Wyk and Smith, 1999). Both GH and IGF-1 would account for all the stages of differentiation by stimulating the growth plate through the specific receptors (Robson *et al.*, 2002). While PTH is intimated involved in the formation and activation of osteoclasts (Aubin and Bonnelye, 2000), it potentially stimulates de novo synthesis of IGF-1 in osteoblasts (McCarthy *et al.*, 1989).

1.6.2. Membranous Ossification

Intramembranous ossification is the other process during fetal development of the mammalian skeletal system in which bone tissue is created. Similar to endochondral ossification, it is an essential process for healing bone fractures (Brighton and Hunt, 1991) and the formation of bones of the skull (Morimoto *et al.*, 1987). The process can be characterized by mesenchymal cells, in the membrane forming osteochondral progenitor cells. They eventually will specialize to become osteoblasts cells. The secretion of bone matrix from the osteoblasts would surround the nearby collagen fibers to become osteocytes. Then, the process of trabeculae formation would develop which then entrap the osteoblasts to form a spongy cell. Cells in the spongy area would further specialize into red bone marrow, while the cells surrounding the developing bones would become periosteum (Hall and Miyake,

1992). Finally, osteoblasts from the periosteum on the bone matrix will produce compact bone (Martin *et al.*, 1998). Previous studies have identified important factors contributing to membranous ossification, including bone-specific transcriptional modulator core binding factor-1 (CBFA-1) (Ducy *et al.*, 1997), Indian hedgehog (Ihh) (St-Jacques *et al.*, 1999), distalless homeobox 5 (Dlx5) (Acampora *et al.*, 1999) and twist homolog (TWIST) (el Ghouzzi *et al.*, 1997). Although these various factors have been shown to regulate membranous ossification, the complex interaction between bone formations in conjunction to endochondral ossification is not fully understood.

1.7. Bone (re)modeling by osteoclasts and osteoblasts

The bone modeling and remodeling processes are quite similar at cellular level. These are mainly based on the interactions between osteoclasts (bone resorbing cells) and osteoblasts (bone forming cells). The remodeling process begins at the quiescent bone surfaces with the appearance of large multinucleated osteoclasts (Vaananen and Horton, 1995). They would attach to the bone matrix tissue to form a ruffled border at the interface between the bone and osteoclasts layer, creating an isolated microenvironment. Subsequently, the osteoclast would acidifies the microenvironment and dissolves the organic and inorganic matters of the bone matrix (Vaananen *et al.*, 2000). This process is known as bone resorption. With the resorptive process ceased, osteoblasts would appear in the same surface. Osteoblasts are derived from mesenchymal stem cells found in the bone marrow, periosteum and soft tissue. They would deposit osteoid to mineralize the surface and form new bone, while some of these osteoblasts are encapsulated in the osteoid

matrix to differentiate into osteocytes. Remaining osteoblasts continue to synthesize bone until they eventually stop and transform into quiescent lining cells that completely cover the newly formed bone surface. These lining cells are highly interconnected with the osteocytes in the bone matrix through a network of canaliculi (Lian and Stein, 2001).

The close relationship between osteoclasts and osteoblasts in the remodeling process has been known as "Basic Multicellular Unit" (BMU) (Frost, 1964). This concept indicates that a coupling mechanism must exist between formation and resorption, with the mechanism remains uncertain. The organization of the BMU's in cortical and trabecular bone differs, but these differences are mainly morphological rather than biological. In the cortical bone, the BMU would form a cylindrical canal thatgradually burrows through the bone, with the tip (consisting of osteoclasts) digging circular tunnels (cutting cone) in the dominant loading direction (Petrtyl *et al.*, 1996). This is followed by filling of the tunnel by osteoblasts (closing cone) to produce a (secondary) osteon of renewed bone (Parfitt, 1994). With this repeated process, about 2% and 5% of cortical bone can be remodeled each year (Parfitt, 1994). Remodeling process of trabecular bone in contrast, occurs mainly on the surface. With much larger surface to volume ratio, this process is intensely more active than cortical bone, with the rate up to 10 times higher (Einhorn and Lee, 2001). Similarly, osteoclasts would be the first to migrate into the process. Like the process in cortical bone, this is quickly followed by bone formation. As the trabecular BMU is a half cortical BMU, the resulting structure that is formed is called a trabecular osteon or hemi-osteon (Frost, 1986, Kassem *et al.*, 1992). The cellular activities of osteoclasts and osteoblasts in modeling are basically similar to

those in remodeling. However, in this case formation and resorption are not balance, the activities of osteoclasts and osteoblasts can also be completely uncoupled thus leading to changes in the micro-architecture of the trabecular bone. . Indeed, complete unloading may cause resorption that is not followed by formation (Mosekilde, 1990). Nevertheless, bone formation does not necessarily be preceded by resorption. It was also observed that the lining cells at the bone surface can transform back to bone forming osteoblasts (Chow *et al.*, 1998, Dobnig and Turner, 1995).

1.8. Factors Affecting Osteoblasts Regulation

The process of initiation, differentiation and apoptosis of osteoblasts are affected by many systemic hormones and local factors. These include gonadal steroids (estrogen and androgens), PTH/PTHrP, bone morphogenetic protein (BMP), and fibroblast growth factors (FGFs), that have been widely discussed and reviewed (Komori, 2006, Lian *et al.*, 2006, Matsubara *et al.*, 2008, Nishimura *et al.*, 2008, Tenta *et al.*, 2006). Other than these, many new confounding regulators are continuously discovered.

1.8.1. Insulin-like Growth Factor Type 1 (IGF-1)

GH can affect bone size and mass through stimulating IGF-1 production in liver and bone. GH exerts many of its effects by stimulation of IGF-1 (Sotiropoulos *et al.*, 2006). IGF-1 is a small polypeptide with homology to pro-insulin that is produced

by a number of cell types. IGF-1 signals via the IGF-1 receptor (IGF-1R), engaging extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (Pl3K) pathways through Src homology 2 domain-containing proteins and insulin receptor substrates-1 and 2 (Dietrich *et al.*, 2000, LeRoith, 2000). The function of IGF-1 was shown to induce proliferation of MC3T3 osteoblast-like cells (Merriman *et al.*, 1990) and is an important survival factor for many mammalian cell types, including osteoblasts. In mice, targeted overexpression of IGF-1 accelerates new bone formation and increases the rate at which matrix is mineralized (Zhao *et al.*, 2000), whereas osteoblast-specific disruption of the IGF-1R causes markedly impaired mineral apposition rate and increased mineralization lag time (Zhang *et al.*, 2002). However, it has been difficult to precisely define discrete actions of each of these factors.

1.8.2. Interleukin-6 (IL-6)

This is a multifunctional cytokine that can activate target genes involved with proliferation, differentiation and apoptosis in a variety of cells (Kishimoto *et al.*, 1995). The effect occurs after binding to its transmembrane receptor (IL-6 receptor or alpha receptor) and the signal inducer gp130 (beta receptor). This receptor-ligand interaction activates the receptor-bound Janus kinase (JAK). The phosphorylation of the tyrosine residues of the cytoplasmic tail of the beta receptor by JAK will be followed by recruitment of signal transducers and activator of transcription (STAT). Further phosphorylation of STAT would generate the formation of STAT-protein dimers that migrate to the nucleus and initiate gene transcription. In bone, the production of IL-6 can be found in many types of cells,

such as osteoblasts, mononuclear phagocytes, endothelial cells, chondrocytes, fibroblasts and lymphocytes (Heymann and Rousselle, 2000, Littlewood *et al.*, 1991). An imbalance or over-expression of IL-6 can result to osteoporosis, rheumatoid defects and accelerated bone turnover (Ershler and Keller, 2000, Hoyland *et al.*, 1994). In addition, transgenic mice with over-expression of IL-6 would resulted in severe skeletal defects including osteopenia, defective ossification and abnormality in the growth plate (De Benedetti *et al.*, 2006). These findings indicate the importance of IL-6 as a regulator of bone remodeling and its role toward bone homeostasis. However, extensive studies would be needed to better understand the mechanism of action, as the relation between osteoblasts and IL-6 remains unknown.

1.9. Current Hypothesis on the Etiology of AIS

1.9.1. Abnormalities in Skeletal Development

Up to date, there is no single explanation for the cause of idiopathic scoliosis. Theories on abnormal bone growth and skeletal disproportion have been related to the pathogenesis of AIS (Guo *et al.*, 2003). Guo *et al.* (2003) conducted a study to demonstrate such observation. It was reported that the AIS girls had longer vertebral bodies and shorter pedicle height between T1 and T12 vertebrae when compared with normal controls. Moreover, another study (Siu King Cheung *et al.*, 2003) showed that many differences in anthropometry were revealed in a comparison of 598 AIS girls with 307 healthy controls. Such significant differences included shorter body height, corrected height, arm span, sitting height and leg height. Burwell *et al* (2006, , 2008a, , 2008b, Nicolopoulos *et al.*, 1985) proposed that the

difference in AIS is significant based on anthropometric measurements.

1.9.2. Genetic Factors

Although specific etiology of AIS remains undefined, the role of hereditary or genetic factors in the curvature development is widely accepted (Axenovich *et al.*, 1999, Chan *et al.*, 2002, Justice *et al.*, 2003, Kesling and Reinker, 1997, Riseborough and Wynne-Davies, 1973, Salehi *et al.*, 2002). Clinical observations and longitudinal studies have documented a higher incidence of scoliosis occurring within families and relatives, as opposed to the general community (Miller, 2007, van Rhijn *et al.*, 2001). It has been consistently shown that the occurrence of scoliosis is higher in monozygous twins (Kesling and Reinker, 1997, Weiss, 2007) and those with reported scoliotic first degree relatives (Czeizel *et al.*, 1978, Miller, 1999, Riseborough and Wynne-Davies, 1973). Despite the documentation on the familial prevalence in AIS, the mode of inheritance remains subjective. In a wide variety of population studies, some authors suggested this occurrence is an autosomal dominance or sex-linked inheritance. However, other states the inheritance would be multifactorial in explaining the wide spectrum of scoliosis occurrence among the individuals of affected families.

In early studies, Wynne-Davies *et al.* (1968) screened first to third degree relatives of 114 AIS patients. Their findings indicated a possible dominant or multiple gene inheritance for the occurrence of scoliosis. In addition, Robin *et al.* (1975) showed that scoliosis occurred in 15 members of a family for three successive generations. Studies associated with twins often showed the presence of idiopathic scoliosis (Carr,

1990, McKinley and Leatherman, 1978, Weiss, 2007). In contrast, De George and Fisher (1967) conducted a questionnaire study of 1378 AIS patients for determining whether there is a predictable mode of inheritance. The study concluded that there was not enough evidence on the mode of AIS being inherited. However, this study had not examined the X-rays of all the scoliotic family members, who might be unaware of having mild scoliosis. In a more recent study, Miller *et al.* (2006) performed a genomic screening and statistical linkage analysis of 202 families with at least two individuals diagnosed with idiopathic scoliosis. In the linkage analysis, several candidate regions for AIS, with one reported chromosome similar to a previous report, was identified (Chan *et al.*, 2002). However, the author failed to find any disorder among the structural genes encoding for elastin and type I collagen. Hence, previous reports (Sharipov *et al.*, 2006, Zaidman *et al.*, 2006) on the association between familial AIS and aggrecan gene remained questionable.

Although the role of genetic factors in the development of AIS is widely accepted, the specific mode of inheritance has not been resolved. More recently, analysis on sporadic idiopathic scoliosis was conducted in search of a possible candidate gene (Cheng *et al.*, 2007, Giampietro *et al.*, 1999). However, difficulties in clinical definition and environmental factors increase the variability in the onset of the disease. Most importantly, it is clear that a larger patient population would be needed to possibly identify the role of genetic factors toward the etiopathogenesis of this disorder.

1.9.3. Neuromuscular Impairment

Clear evidences on abnormal somatosensory-evoked potentials (SSEP) (Cheng *et al.*, 1998a, Harrington, 1977) and tonsilar ectopia from magnetic resonance imaging (MRI) (Cheng *et al.*, 1999b, Chu *et al.*, 2007) have been reported. Among the 135 subjects diagnosed with AIS, it was found that about 4% would develop syringomyelia, with a higher incidence in males. Toward low-lying cerebellar tonsil, Chu *et al.* (2007) reported that 42% of the 69 AIS subjects had tonsilar trips below the basionopisthion reference line (BO line). Likewise, the regional brain volume was significantly different between AIS patients and age-matched control (Liu *et al.*, 2008). In this study, 10 out of 22 measured regions were larger in AIS patients than control, while the remaining regions were smaller. Similarly, Chu *et al.* (2006) reported spinal cord tethering in severe AIS patients that resulted from the immense curvature. This suggested a disproportionate growth between the neural system and skeletal development of AIS. These findings provide a possible explanation for the poor performance in visual testing and impaired balance reported in AIS patients.

It is a common understanding that stability of the spine depends on the interaction between intrinsic factors (bone, facet joints and ligaments) and extrinsic factors (muscle force, elasticity of tendons) (Hosman, 2003). Many diseases affecting muscles or their functions tend to associate with the occurrence of secondary scoliosis deformity (Aprin *et al.*, 1982, Hsu, 1983, James, 1956, Robin, 1972). Likewise, muscle imbalance has been associated with the etiology of AIS (Fidler and Jowett, 1976, Riddle and Roaf, 1955). As far as the 19th century, Adams (1882)

conducted a postmortem investigation of AIS patients and found muscle hypertrophy at the convex side and atrophy at the concave side of the curvature. This finding suggested muscle force could play a role in preventing spinal progression, rather than inducing it. Histochemical studies have also been conducted on the paraspinal muscle in AIS patients (Cheung *et al.*, 2004). In France, a study demonstrated that the middle and deep dermis muscle of AIS was significantly different from normal healthy subjects (Echenne *et al.*, 1988). Similarly, Cheng *et al.* (2001) found a lower expression of glycoproteins in the iliac crest biopsies from AIS patients. Both studies demonstrated a difference in anatomical structure in AIS patients. In another study, Moreau *et al.* (2008) isolated messenger RNA from paraspinal muscle intraoperatively from AIS patients. Expression analysis showed that the left restricted genes were more expressed on the right side. This observation can explain the prevalence of the right thoracic curve over the left curve. However, to an extent, the impaired muscle development is likely a secondary cause of spinal deformity, rather than a primary one.

1.9.4. Hormone Dysfunction

Many related issues have linked hormone dysfunction with the etiopathogenesis of AIS. Hormones studied include growth hormone, leptin and melatonin. A study by Wilner *et al* .(1976) found higher plasma growth hormone and serum somatomedin A levels in AIS girls than age-matched controls. The same group (Dymling and Willner, 1978) found that treatment of AIS patients with growth hormone over a 10-week period would create a higher curve progression and on the termination of treatment, curve progression could be deferred. A similar finding on

associating growth hormone with AIS patients was conducted by Skoglund and Miller (1980). They found a significantly higher growth hormone release mechanism in prepubertal girls diagnosed with AIS. On the contrary, a study in Sweden (Ahl *et al.*, 1988) did not detect any difference in the growth hormone level of AIS girls in pubertal stages 3 and 4 from the matched control. However, difference was found between AIS girls at pubertal stage 2 and the control. Qiu *et al.* (2007b) revealed that the growth hormone receptor did not appear to be a predisposing gene or disease modifier gene in AIS. These studies raised further concerns on whether growth hormone does play a crucial role in the etiology of AIS. Another hormone of great interest toward AIS is leptin. A study reported a marked decrease in circulating leptin level in AIS girls from a comparison of 120 AIS girls with 80 healthy controls (Qiu *et al.*, 2007c). In analyzing the results, leptin was positively associated with body weight, BMD, body mass index (BMI) and other growth parameters. Similarly, Burwell *et al.* (2008a) hypothesized that leptin would influence the BMI and skeletal maturation in AIS. However, there is still a lack of related in-depth studies on the relationship between leptin and the etiopathogenesis of AIS. Most recently, melatonin deficiency has been widely linked with the occurrence of AIS. Melatonin is a hormone secreted primarily from the pineal gland. Further discussion on this hormone would be given in the following section.

1.10. Melatonin

N-acetyl-5-methoxytryptamine (melatonin) is a hormone secreted primarily from the pineal gland. It was first discovered by Aaron Lerner and his colleagues in 1958

(Lerner *et al.*, 1960). Melatonin (Figure 1.4) is a hormone found in all living organisms, including non-mammalian vertebrates, some invertebrates, and in many plants (Hardeland and Poeggeler, 2003, Pandi-Perumal *et al.*, 2006). It is biosynthesized from biochemical pathway (Figure 1.5). The amino acid tryptophan is first converted by tryptophan hydroxylase to 5-hydroxytryptophan. Next, 5-hydroxytryptophan is further decarboxylated to serotonin. The synthesis of melatonin by serotonin is governed by two main rate-limiting enzymes; arylalkylamine-*N*-acetyltransferase (AA-NAT) and hydroxyindole-*O*-methyltransferase (HIOMT), which is mainly produced in the pineal gland (Axelrod and Weissbach, 1960, Coon *et al.*, 1995). Other organs producing melatonin include retina, extraorbital lacrimal gland, Harderian gland, gastrointestinal tract, blood platelets and bone marrow cells (Pandi-Perumal *et al.*, 2006). Metabolism of melatonin occurs mainly in the liver, in which it is hydrolyzed to 6-hydroxymelatonin. Then, it is excreted in the urine as 6-sulfatoxymelatonin (Lynch *et al.*, 1975).

The synthesis and release of melatonin by the pineal gland are stimulated by darkness and inhibited by light (Lowe *et al.*, 2000). Without light, the unpolarized photoreceptors release norepinephrine to highly activate a number of adrenergic receptors in the pineal gland (Pangerl *et al.*, 1990). This onset stimulates the AA-NAT enzymatic activity to synthesis and release melatonin into the bloodstream, by passive diffusion (Pangerl *et al.*, 1990). In addition, light does not cause the timing but entrains it (Brzezinski, 1997). The function of light can only modify the diurnal cycle for melatonin secretion, or abruptly suppresses the production (Lewy *et al.*, 1980). The melatonin threshold is between 200-400 lux, while intensity higher

than 600 lux would cause maximal inhibition (McIntyre *et al.*, 1989). However, seasonal variations of a longer summer (Lowe *et al.*, 2000) and increase in the age of an individual (Lynch *et al.*, 1975) would also cause drastic depression in melatonin production.

In 1994, the cloning of melatonin receptors in mammals was first reported (Reppert *et al.*, 1994). In mammals, mainly two types of membrane-bound melatonin-binding sites have been identified: ML1 (high affinity) and ML2 (low affinity) receptors. Activation of ML1 receptors is responsible for the inhibition of adenylate cyclase activity in target cells (Ebisawa *et al.*, 1994, Turek and Gillette, 2004). With the use of polymerase chain reaction, two further subtypes of ML1 receptor were cloned in humans: MT1 and MT2 receptors (Reppert *et al.*, 1995, Reppert *et al.*, 1994). These two membrane receptors mediate several major actions of melatonin. Belonging to the G-protein coupled receptors superfamily, MT1 receptors activate protein kinase C-b. At the same time, the MT2 receptors would inhibit the guanylate cyclase pathway and stimulate protein kinase C (Reppert *et al.*, 1995, Reppert *et al.*, 1994). In the example of a rodent's brain, the widespread occurrence of MT1 receptors indicates the majority for melatonin-binding sites. Likewise, the lower expressions of MT2 receptors demonstrate a minor action, and independence to photoperiodic response (Reppert *et al.*, 1995). This hormone is involved in many regulations including embryo development (Chan and Ng, 1994a, Chan and Ng, 1994b), cardiac monitoring (Ghosh *et al.*, 2007), circadian rhythm (McArthur *et al.*, 1997, Redman *et al.*, 1983), sexual maturation (Silman *et al.*, 1979, Sizonenko *et al.*, 1985), aging (Armstrong and Redman, 1991, Sandyk, 1990), tumor malignancy (Hill and Blask, 1988, Lissoni *et al.*, 1994, Panzer, 1997, Shiu *et al.*,

2003, Yang *et al.*, 2007) and bone growth (Ladizesky *et al.*, 2003, Nakade *et al.*, 1999, Ostrowska *et al.*, 2003). In this study, it will focus on the relation of melatonin with bone development.

1.10.1. Melatonin and Bone-related Studies

In the early 90's, melatonin was believed to be associated with bone metabolism. Although without any compelling evidence, it was suggested that melatonin might be a possible remedy in reducing the rate of osteoporosis in menopausal women (Sandyk *et al.*, 1992). The idea originated from the observation that osteoporosis occurred most rapid at menopause, in which melatonin and other hormones decline briskly in level with dramatic decrease in bone turnover rate. Other studies have suggested an association of age progression and decreasing level of melatonin (Grad and Rozencwaig, 1993, Rozencwaig *et al.*, 1987). In these studies, the use of melatonin was proposed as a possible treatment for improving bone health. Some studies suggested prescription of melatonin for enhancing bone growth in spinal fusion (Abdel-Wanis and Kawahara, 2002).

In a study related to medication for preventing osteoporosis at the menopausal age, Ladizesky *et al.* (2001)used ovariectomized rats to assess the changes in urinary deoxypryridinoline, calcium excretion, blood levels of calcium, phosphorous, and bone alkaline phosphatase. In addition, they evaluated BMD, BMC and BA of the entire skeleton after melatonin treatment (250 μg/mL) through drinking waters. After 30 days, the untreated group showed an increase in urinary deoxypryridinoline that was not found in the melatonin-treated group. Serum phosphorous and bone

alkaline phosphatase had increased significantly in the melatonin-treated groups at 15 days after surgery. However, the BMD, bone mineral contents (BMC) and bone area (BA) were decreased in both the melatonin-treated group and untreated group 60 days after surgery. In another study (Ladizesky *et al.*, 2006), male rats were treated with melatonin or methylprednisolone, a synthetic glucocorticoid agent, or with a combination of the two. After 10 weeks, rats treated with either melatonin or methylprednisolone had significantly stimulated BMD, BMC and BA. From these studies, it is evident that melatonin can reduce bone resorption and possibly prevent bone damage.

1.10.2. Pinealectomized and Bipedal Scoliotic Animal Model

In the early 1959, Thillard first reported that the removal of the pineal gland, also known as pinealectomy (PINX), can produce scoliosis in chickens (Thillard, 1959). As introduced previously, the main secretory product of the pineal gland is melatonin. By removing the pineal gland from chicken, they could produce post-operative spinal curvature, similar to that in AIS patients. This phenomenon brings forth a hypothesis which associates melatonin with the occurrence of AIS.

However, the occurrence of scoliosis varied widely from PINX alone (Table 1). PINX alone was not able to induce scoliotic curvature in rats unlike the avians (O'Kelly *et al.*, 1999). This led to the theory that the quadrupedal movements of the rats do not create the much needed pressure as the bipedal movements of the chicken. Machida *et al.* (1999) decided to confirm this theory. In this study, they created a few groups of rats: sham-operated bipedal, PINX quadrupedal, PINX bipedal and

PINX with implanted melatonin pellets. Three months after surgery, only PINX bipedal rats developed scoliosis similar to that of PINX chicken. Importantly, treatment with the melatonin pellet prevented 90% (9/10) of the PINX bipedal rats from developing scoliosis. However, Cheung *et al.* (2005) were not able to show the relation between pinealectomized non-human primates and the development of scoliosis. In the 29 months post-operative follow-up, 10 out of 18 rhesus monkeys have a significant loss in melatonin secretion, while none developed scoliosis. Yet, this finding was criticized for the limited area of movement to allow the monkeys to move freely or stand upright naturally on inducing the vertebral stress (Reiter *et al.*, 2007). Nevertheless, these findings do suggest that postural processes and melatonin deficiencies are crucial factors in weakening the vertebral column to induce scoliosis formation.

1.10.3. Natural Melatonin Deficient Model, C57BL6J

Recently, a study with melatonin-deficient mice (C57BL/6J) showed that the lack of melatonin in rodent would be associated with the development of scoliosis. It has been shown that the C57BL/6J strain mice have a natural melatonin deficiency due to the reduce enzymatic rate of melatonin synthesis by the knock-out of the AA-NAT gene during initial mating (Ebihara *et al.*, 1986). The mice lack the AA-NAT gene, a key enzyme for the production of melatonin from serotonin, exhibiting a depressed melatonin level in plasma and pineal gland compared with other inbreed species of mice. In addition, numerous studies have shown this mouse to have the lowest BMD among the inbred strains of mice (Chen and Kalu, 1999, Dimai *et al.*, 1998, Richman *et al.*, 2001, Sheng *et al.*, 1999, Turner *et al.*, 2001). Hence, this makes it

an ideal model for assessing the effect of melatonin with bone formation *in vivo*.

Machida *et al.* (2006) attempted to study the relation of melatonin-deficiency and the occurrence of scoliosis by utilizing this strain of mouse together with the surgically-induced bipedal to assess the rate of development of scoliosis and the effect of daily injections of melatonin (8 mg/kg) in reversing scoliosis development. After treatment for 5 months, the mice were sacrificed and the spine was examined by X-ray and 3D CT. Nearly 100% of the melatonin-deficient bipedal mice (Figure 1.5) developed spinal deformity, while a quarter of the quadrupedal model naturally developed scoliosis (Table 1.2). In both groups of mice, daily melatonin treatment prevented scoliosis development.

In line with the previous study, the same team extended their study with this unique mouse (Oyama *et al.*, 2006). They removed the forelimbs of these mice, without performing PINX, and produced scoliosis at a higher rate (Table 1.3). As the control, they removed the forelimbs of another type of mice, C3H/HeJ (a melatonin proficient mice), without pinealectomy. The rate of scoliosis occurrence was two-fold less than the bipedal C57BL/6J mice. However, once PINX was also performed in the bipedal C3H/HeJ mouse, the prevalence of scoliosis rose to 70%. The finding demonstrated that spinal malformations would occur in combined melatonin deficiency and bipedal amputation.

1.10.4. Effects of Melatonin in Osteoblasts

There are very few reports showing the effect of melatonin in osteoblasts in the

current literatures (Nakade *et al.*, 1999, Roth *et al.*, 1999, Satomura *et al.*, 2007). Roth *et al.* (1999), were first to report the effect of melatonin toward two rodent osteoblastic cell lines (MC3T3-E1 and ROS 17/2.8). The results suggested that melatonin may play little or no role in the osteoblasts' proliferation. However, melatonin promoted cell differentiation and stimulated mineralization. In contrast, Nakade *et al.* (1999) reported melatonin's ability to enhance proliferation in normal human osteoblasts. However, there was no effect on differentiation. Because these two findings contradict each other, the effect of melatonin on osteoblasts remains controversial.

In a more complete study, Satomura *et al.* (2007) demonstrated the dose-dependent stimulation of melatonin in proliferation, alkaline phosphatase activity and rate of mineralization in normal human osteoblasts. Likewise, the presence of MT1 expression was confirmed by real-time polymerase chain reaction (RT-PCR) and western blotting analysis. Also, the author found a linear correlation with an increase in age and decrease in MT1 expression. In the same study, intraperitoneal injections of melatonin (100 mg/kg/day) were given to a group of mice for 21 days to assess bone formation by immunohistology. Interestingly, melatonin was able to increase the bone volume and newly formed cortical bone in the femurs of the mice. As for osteoblasts are derived from mesenchymal stem cells (MSC), the study (Radio *et al.*, 2006) demonstrated an increase in alkaline phosphatase (ALPase) activity after melatonin treatment. These observations (Nakade *et al.*, 1999, Radio *et al.*, 2006, Roth *et al.*, 1999, Satomura *et al.*, 2007) further suggested the enhancement effect of melatonin on bone cells.

1.10.5. Melatonin in AIS

At present, the causes of AIS remain uncertain and are probably multifactorial. There is currently no proven prognosis to identify children or adolescents with AIS. Current reports targeted associations with the pathogenic attribute (Hung *et al.*, 2005) rather than the etiological factors. With the many suggestions concerning the etiology of AIS, melatonin may play an essential role in the pathogenesis of scoliosis.

The resemblance in spinal deformity and reduced melatonin level of PINX chicken led to the relation of melatonin-deficiency and the occurrence of AIS (Bagnall *et al.*, 1999, Dubousset and Machida, 2001, Machida *et al.*, 1995, O'Kelly *et al.*, 1999). Machida *et al.* (1996) conducted a study to determine any difference in serum melatonin level between AIS patients and normal healthy control. Significant difference was found in a comparison of progressive AIS patients with control and non-progressive AIS patients. However, there was no difference in the serum melatonin level between the normal control and non-progressive AIS patients. Likewise, other studies on comparing the melatonin level in serum (Bagnall *et al.*, 1996, Brodner *et al.*, 2000) and urine (Fagan *et al.*, 1998, Hilibrand *et al.*, 1996) showed no difference between AIS patients and normal control. However, unlike the study of Machida *et al.* (1996), these studies might not show any difference in generalizing both progressive and non-progressive AIS patients as one group. Also, Bagnall *et al.* (1996) suggested the difference in melatonin level might be observed during the onset of scoliosis. Thus, the data on human melatonin level are controversial and melatonin deficiency as a causative factor in the etiology of AIS

seemed unsupported.

In order to speculate the possible relation of melatonin with AIS, there might be a dysfunction of the melatonin-signaling pathway in AIS. Moreau *et al.* (Azeddine *et al.*, 2007, , 2004) examined osteoblasts from 41 AIS patients and 17 control patients. Primary osteoblast cultures were prepared from bone biopsies obtained intraoperatively during spine surgeries. Melatonin and 5'-guanylimidodiphosphate (Gpp(NH)p), a guanosine-5'-triphosphate (GTP) analogue, were tested in the cells for their ability to block cyclic adenosine monophosphate (cyclic AMP) accumulation induced by forskolin. In parallel, the results showed that melatonin signaling was clearly impaired varying in osteoblasts isolated from AIS patients. A new classification of patients with AIS can be divided into three distinct groups, based on their responsiveness to melatonin. In addition, the study suggested that the abnormalities in Gi protein function could be the cause in the occurrence of AIS.

It is well-known that the mutation of genes might be implicated in causing a signal dysfunction in the melatonin signaling pathway. Qiu *et al.* (2007a) found that melatonin 1B receptor (MTNR1B) gene polymorphism was associated with the occurrence of AIS (Qiu *et al.*, 2007a). However, there was no association with the curve severity. Evidently, melatonin may play a key role in the pathogenesis of AIS. Undoubtedly, the diurnal variations in the melatonin level and circadian rhythm of melatonin secretion would be important. However, the question remains on the causative effect of melatonin in the etiology of AIS being primary or secondary.

Figure 1.1. Radiological image of right thoracic idiopathic scoliosis.

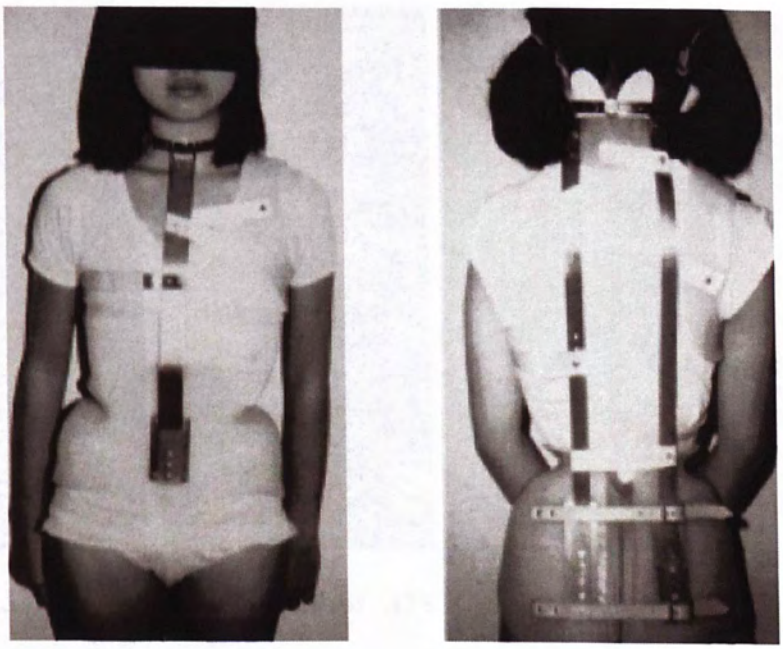

Figure 1.2. Illustration of an AIS patient treated with a Milwaukee brace.

(Image adopted from Tang SP *et al.*2003 (Tang *et al.*, 2003))

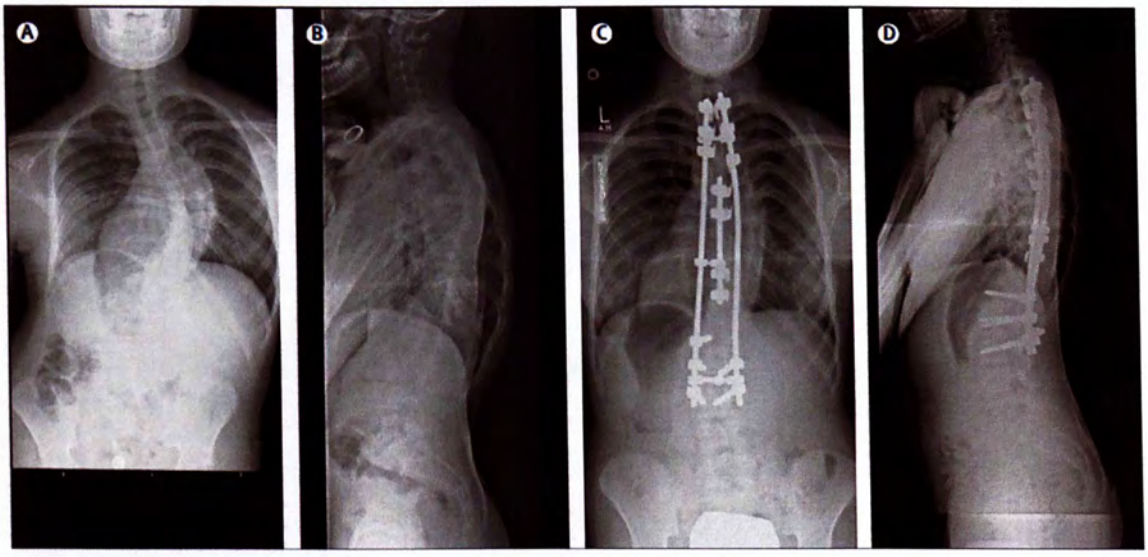

Figure 1.3. Radiograph images of AIS patients before and after surgical correction. A) Pre-operative standing posterior view; B) Pre-operative standing lateral view; C) Post-operative standing posterior view; D) Post-operative standing lateral view.

(Image adopted from Weinstein *et al.*2008 (2008))

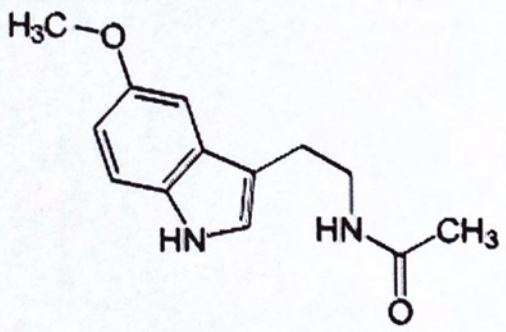

Figure 1.4. Structure of Melatonin

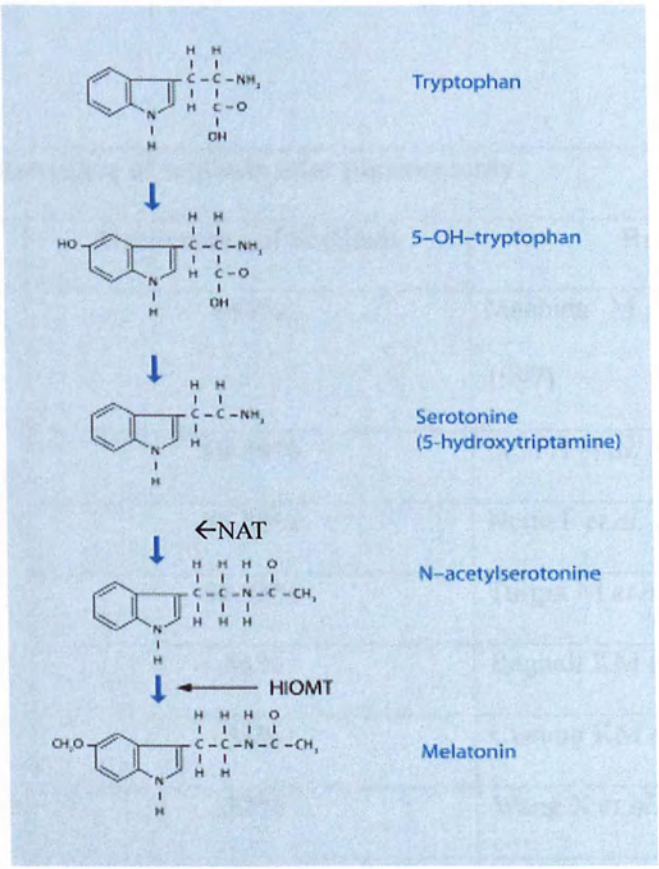

Figure 1.5. Biosynthesis of Melatonin. N-acetlytransferase (NAT) and hydroxyindole-O-methyletransferase (HIOMT) are key enzymes in the melatonin biosynthetic pathway. (Image adopted from Grivas TB *et al.* 2007 (2007))

Table 1.1. Occurrence of scoliosis after pinealectomy		
	Occurrence of Scoliosis	**Report**
Chickens	**100%**	Machida M *et al.* (1995, , 1997)
	60-80%	Inoh H *et al.* (2001)
	50-80%	Nette F *et al.* (2002)
	50-58%	Turgut M *et al.* (2003)
	56%	Bagnall KM *et al.* (1999)
	54%	Cheung KM *et al.* (2003)
	52%	Wang X *et al.* (1998)
	48%	O'Kelly C *et al.* (1999)
Bipedal Rats	**100%**	Machida M *et al.* (1999)
Monkeys	**0%**	Cheung KM *et al.* (2005)
Salmons	**82%**	Fjelldal PG *et al.* (2004)

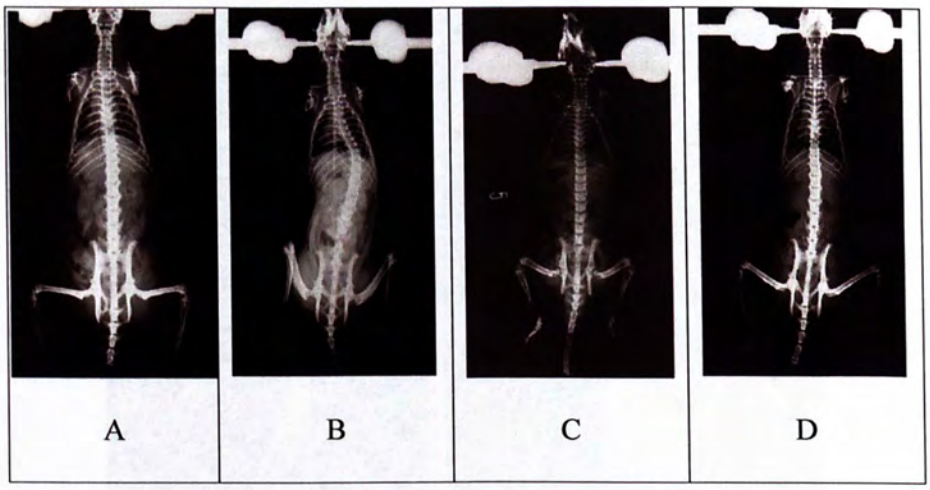

Figure 1.6. Occurrence of spinal deformity in rats under different conditions. A) A straight spine after bipedal operation, B) Severe scoliosis of the spine after pinealectomy (PINX) and bipedal operation, C) A straight spine after PINX operated quadrupedal mouse, and D) A straight spine after melatonin administration to a PINX operated bipedal rat. (Image adopted from Machida *et al* 1999 (1999))

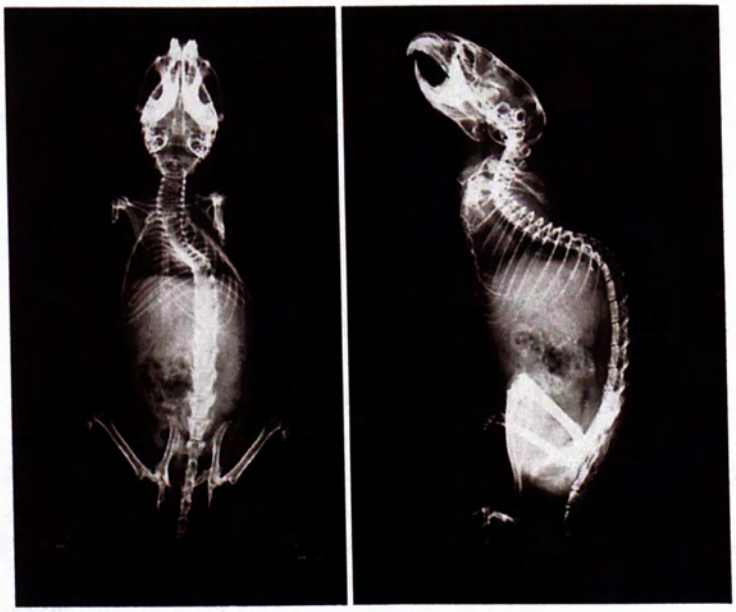

Figure 1.7. Occurrence of right thoracic scoliosis in a bipedal mouse. (Image adopted from Machida *et al.* 2006 (1995))

Table 1.2. Experimental scoliosis in melatonin-deficient C57BL/6J mice with an intact pineal gland (Data reported and adopted from Machida M *et al.* 2006 (2006))

	Occurrence of Scoliosis	
	No Melatonin Treatment	**Melatonin Treatment (8mg/kg)**
Bipedal C57BL/6J mice	**97% (29 out of 30)**	**0% (0 out of 30)**
Quadrupedal C57BL/6J mice	**25% (5 out of 20)**	**0% (0 out of 20)**

Table 1.3. Effects of bipedal operation with or without pinealectomy on the induction of scoliosis in C3H/HeJ and C57BL/6J mice (Data reported and adopted from Oyama J *et al.* 2006 (2006))

	Occurrence of Scoliosis		
	Bipedal C3H/HeJ mice	**Bipedal C57BL/6J mice**	**Bipedal and PINX C3H/HeJ mice**
Single site (%)	**25.0**	**64.3**	**70.0**
Double site (%)	**None**	**14.3**	**50.0**

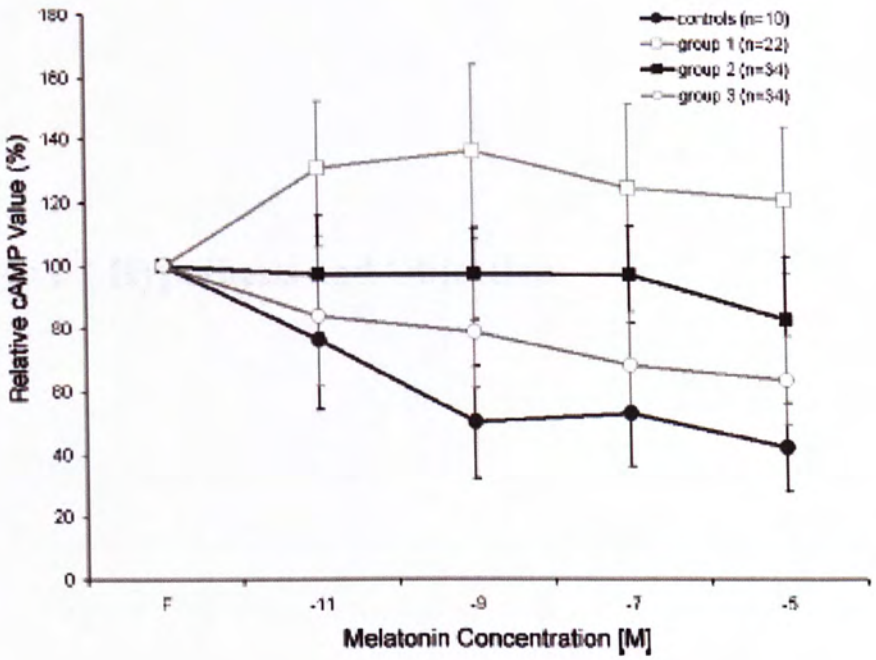

Figure 1.8. Evaluation of melatonin inhibitory effect on adenylyl cyclase activity in normal human and AIS osteoblasts. The AIS subjects are further subgrouped based on their responsiveness toward melatonin; Group 1, Group 2 and Group 3. Results are represented mean ± SEM. (Graph adopted from Azeddine B *et al.* 2007 (2007))

Chapter 2 Hypothesis and Objectives

2. Hypothesis and Objectives

2.1. Study Hypothesis

Adolescent idiopathic scoliosis is the major type of spinal deformity abnormal in bone growth and BMD in adolescent girls. Although different treatments have been applied for the girls with different curve severity, they are not satisfactory. This is mainly due to the lack of understanding on the etiology of AIS. Among different possible causes of AIS, melatonin should play an important role on the etiopathogenesis. The recent finding on the melatonin signaling pathway dysfunction might suggest the heterogeneity in AIS population. Taken together, it is hypothesized that there is an abnormality in the response of osteoblasts from AIS girls to melatonin. The aim of this study is to investigate the cellular activity of osteoblasts from AIS girls' and it's responses to melatonin.

2.2. Objectives

In order to to address the study hypothesis, the following three objectives are:

1. To study the anthropometric parameter and BMD of girls with severe AIS in comparison with healthy female adolescent controls.
2. To determine the effect of melatonin on proliferation and differentiation of osteoblasts isolated from intraoperative bone biopsies of AIS patients.
3. To detect the expression of melatonin receptors on osteoblasts isolated from intraoperative bone biopsies of AIS patients in comparison with healthy normal control.

Chapter 3 Study on the Anthropometric Parameters and Bone Geometry of Girls with Severe AIS

3.1. Introduction

Adolescent idiopathic scoliosis mainly occurs during peri-puberty or at the stage of rapid growth spurt of a child prior to skeletal maturation. Idiopathic scoliosis can be characterized of having a spinal curvature malformation with vertebral rotation, and possibily lordosis. Earlier studies reported that AIS patients have significantly longer arm span, taller in height and other body segmental lengths than those of healthy controls after the onset of puberty (Burwell *et al.*, 2008b, Cheng *et al.*, 1996, Willner, 1975). Throughout puberty, a lower body weight and BMI has consistently been recorded from AIS girls (Burwell *et al.*, 2008a, Qui *et al.*, 2008, Siu King Cheung *et al.*, 2003). In a cross-sectional study, 621 girls with AIS were studied for the association of bone mass with anthropometric parameters and bone turnover (Cheung *et al.*, 2006). The results showed that the AIS patients have longer and taller anthropometric parameters, generalized osteopenia and 38.6% higher bone alkaline phosphatase (bALP) than the controls. The study concluded that the abnormally faster growth spurt and greater bone turnover of AIS patients might lead to bone dimension increments. A MRI study have shown disproportional or asynchronous growth of the anterior and posterior thoracic vertebral column in AIS patients (Guo *et al.*, 2003). Significant positive correlation between the scoliosis severities with the ratio of differential thoracic growth of the column was also found. The disproportionate growth of the vertebral body in AIS patients was proposed to be contributed mainly by active endochondral ossification, with a relative decrease on the circumferential growth through membranous ossification.

Also, recent studies showed osteopenia occurrence in AIS girls through measurement

using DXA (Cheng and Guo, 1997, Cheng *et al.*, 2000, Cheung *et al.*, 2006) and peripheral quantitative computed tomography (pQCT) (Cheng *et al.*, 2000). In these studies, it was shown that the AIS girls have lower BMD than healthy individuals both for the areal BMD and volumetric BMD. Within the AIS clinic in Prince of Wales Hospital, approximately 25% of AIS patients followed up were classified as osteopenic (Cheng and Guo, 1997, Cheung *et al.*, 2006, Lee *et al.*, 2005b). However, the above studies only focused on a generalized population of AIS patients with different severities. Thus, it would be of interest to understand whether these abnormalities in anthropometry and BMD could be reflected in a group of AIS patients with demonstrable curve progression. This group of severe AIS would be defined with curvature of Cobb's angle greater or equal to 40 degrees. The objective of the current study was to investigate the anthropometric parameters and bone geometry of the severe AIS girls between the ages of 13 to 20 and to compare the results with age and sex matched healthy control subjects. Importantly, this cross-sectional study would further consolidate the observation of abnormal systemic growth and osteopenia in severe AIS girls.

3.2. Methodology

3.2.1. Recruitment of Subjects

3.2.1.1. Patients with Severe Adolescent Idiopathic Scoliosis

In this study, 224 Chinese girls, between the ages of 13 and 20, with a clinical and radiographic diagnosis of severe adolescent idiopathic scoliosis and had a Cobb angle of at least 40^{o}, were recruited. Patients diagnosed with disease other than idiopathic scoliosis, and those who had scoliosis of congenital, neuromuscular,

metabolic etiology, skeletal dyplasia and known endocrine and connective tissue abnormalities or have taken medications were excluded from this study.

3.2.1.2. Normal Control

In accordance, 160 healthy Chinese adolescents without scoliosis, between the ages of 13 and 20, were recruited randomly from the community. The control subjects were clinically examined, with forward bending test (Bunnell, 1984), by an experienced orthopedics surgeon to confirm the absence of scoliosis. Subjects with suspected spinal curvatures were referred to the scoliosis clinic for further confirmation.

3.2.1.3. Patients Consent

The protocol was approved by the Clinical Research Ethics Committee of the University (Appendix II). Informed consents were given by all the subjects and their parents prior to their entrance into the study (Appendix III-VI).

3.2.2. Evaluation of Curve Severity of Scoliosis

Standard radiography of the spine in the standing position of the coronal and sagittal planes was used for measuring the curve severity. Patients with Cobb's angle greater than 40 degree would be classified as severe AIS. For patients with double or triple curves in the spine, the major curve with the greatest angle would be used for analysis.

3.2.3. Anthropometric Measurements

Anthropometric measurement of body weight, height, and arm span were conducted by using standard methods (Cheng *et al.*, 1996, Cheng *et al.*, 1998b). Subjects wore light garment, without shoes, when measuring the body weight on an electronic balance (Soechnle, Germany). Accuracy was taken to the closest 0.1 kg. Similarly for the height, subjects were measured without shoes by standing upright against a wall-mounted stadiometer, with the head positioned in the Frankfort horizontal plane and the heel against the stadiometer (Technical Service Unit of the University). Accuracy was taken to the closest 0.1 cm. For measurements of arm span, the subject's arm was fully stretched horizontally against a wall-mounted tape for measurement. Accuracy was taken to the closest 0.1 cm. For scoliotic patients, corrected height was computed by adjusting trunk loss using Bjure's formula ($\log y = 0.011x - 0.177$; where y is the loss trunk height (cm) caused by the spinal deformity, and x is the Cobb angle of the primary curve) (Bjure *et al.*, 1968). BMI was measured by weight (kg) divided by square of arm span (m^2).

3.2.4. Measurements of BMD

3.2.4.1. Dual-energy X-ray Absorptiometry (DXA)

DXA (XR-36; Norland Medical Systems, Fort Atkinson, Wisconsin) was used to measure the bilateral proximal femoral neck BMD in the anteroposterior position, according to the manufacturer's instructions. To maintain an anteroposterior position, a foot support was used to maintain a 20° inward rotation of the legs to compensate for femoral neck anteversion. Measurement of femoral BMD was done

with standard techniques described previously (Mazess *et al.*, 1991). In this study, only the bilateral proximal femoral necks were chosen for BMD evaluations. As shown in our previous study (Hung *et al.*, 2005), these sites could representadequately the systemic BMD and osteopenia in AIS patients. The precision error of the femoral neck was within 0.8%. BMD was determined from the measured BMC (g) divided by the detected BA (cm^2) (Fuerst *et al.*, 1998).

3.2.4.2. Peripheral Quantitative Computed Tomography (pQCT)

The current study focused on the mid-shaft BMD which mainly reflects membranous ossification, pQCT (Densiscan 2000, Scanco Medical, Basselsdorf, Switzerland) was used to measure volumetric BMD. The long-term reproducibility of 0.3% was demonstrated in mixed reports (Rüegsegger, 1994). The radius of the patients was positioned in a radiolucent splint anatomically comfortable during the pQCT scanning. For the midshaft of radius, the midpoint was marked on the skin and a radiolucent marker was placed around the radius at the mid-point. By a projectional scout view, the reference line was marked at the radiolucent marker. The cross-sectional area and the cortical bone area of midshaft radius were measured with a single slice of pQCT image, at a scan resolution of 0.3 mm. Technical details on using pQCT for volumetric BMD measurement can be found elsewhere (Cheng and Guo, 1997, Cheng *et al.*, 2000).

3.2.5. Data Analysis

Multiple regression analysis, with adjustments for confounding variables, was used

for the comparison of different anthropometric data and the BMD between the severe AIS girls and controls. All data were expressed as the intercepts, regression coefficients and standard errors. The alpha value was set to 0.05 to be considered statistically significant. SPSS software (version 11; SPSS, Inc., Chicago, IL) was used for statistical analysis.

3.3. Results

3.3.1. Anthropometry

A total of 384 adolescents (224 AIS girls and 160 healthy females) were recruited into this study (Table 3.1). In Table 3.1, the average age of severe AIS girls (16.3 ± 2.0) was similar to the normal control (16.8 ± 2.2) used in this study. The age distribution among AIS and control subjects was similar. The average Cobb's angle of the recruited severe AIS patients was 55.8° (range between 40° to 100°).

Table 3.2 shows the age-adjusted measurements of the arm span and body mass index between severe AIS girls and normal controls. The multiple regression analysis of the BMI in the normal controls showed an intercept at 17.99 kg/m^2 with a standard error of 0.91 kg/m^2. As age increase, the BMI would significantly increase ($p=0.045$) by 1.4 kg/m^2 with a standard error of 0.05 kg/m^2. However, if it is an AIS patient, the BMI would significantly ($p<0.001$) be lowered than those of the control subjects by 1.43 kg/m^2 with a standard error of 0.22 kg/m^2. While for the arm span, the multiple regression analysis in the normal controls showed an intercept at 148.23 cm with a standard error of 3.06 cm. As age increase, the arm span would significantly increase ($p=0.001$) by 0.58 cm with a standard error of 0.18 cm.

However, if it is an AIS patient, the arm span would be significantly longer ($p=0.001$) than the normal controls by 2.56 cm in average with a standard error of 0.75 cm.

3.3.2. BMD of Femoral Neck and Midshaft of Radius

Table 3.3 shows the different measurements of BMD of femoral neck by DXA and midshaft radius by pQCT, respectively. Due to the loading of the curvature, the femoral neck was grouped according to the dominance and convexity. With multiple regression analysis, the intercept of the non-dominant, dominant and convex side of femoral neck BMD in the normal control was 0.38 g/cm^2 with a standard error of 0.05 g/cm^2. While for the BMD on the concave side of the femoral neck, the intercept was 0.39 g/cm^2 with a standard error of 0.05 g/cm^2. The BMD of the femoral neck regardless of the dominance and convexity of the curve would significantly ($p<0.01$) increase by 0.01 g/cm^2 with a standard error of 0.01 g/cm^2 as age increased. With an increase in body weight, the BMD of these parameters of the femoral neck would significantly ($p<0.001$) increase by 0.01 g/cm^2 with a standard error of 0.005 g/cm^2. However in severe AIS patients, the BMD of the non-dominant and dominant side of the femoral neck would significantly ($p<0.001$) be lowered than the normal controls by 0.04 g/cm^2 with a standard error of 0.01 g/cm^2. While for the BMD on convexity of the femoral neck, the severe AIS have a lower BMD on the convex and concave side than the control by 0.04 g/cm^2 (S.E. 0.01 g/cm^2) and 0.03 g/cm^2 (S.E. 0.01 g/cm^2), respectively.

In Table 3.4, measurement on the BMD of the cortical and apparent midshafts of radius was assessed by pQCT. The result was divided into 2 age groups to assess

for the difference maturity: 1) below 16 years old and 2) equal of older than 16 years old. By using multiple regression analysis of the adolescent girls below the age of 16, the intercept of the cortical BMD was 1.24 g/cm^3 with a standard error of 0.07 g/cm^3. With age increase in the normal controls, the cortical BMD of the midshaft of radius would significantly increase by 0.02 g/cm^3 with a standard error of 0.01 g/cm^3 ($p<0.001$). However, there was no significance between the severe AIS subjects and normal controls ($p=0.948$). While for the multiple regression analysis on the matured female adolescents, the intercept of the cortical BMD was 1.46 g/cm^3 with a standard error of 0.03 g/cm^3. And when age increases in the matured normal subjects, the cortical BMD would further increase by 0.01 g/cm^3 with a standard error of 0.01 g/cm^3 ($p<0.001$). However, the AIS girls would have a significant lower BMD ($p=0.05$) than the normal controls by 0.01 g/cm^3 with a standard error of 0.01 g/cm^3. Similarly for the midshaft apparent BMD of the adolescent girls below the age of 16, the intercept of the apparent BMD was 0.87 g/cm^3 with a standard error of 0.09 g/cm^3. However, there was no significant toward the increase in age and the presence of scoliosis ($p>0.05$). While for the multiple regression analysis on the matured female adolescents, the intercept of the apparent BMD was 0.94 g/cm^3 with a standard error of 0.05 g/cm^3. And when age increases in the matured normal subjects, the apparent BMD would further increase by 0.01 g/cm^3 with a standard error of 0.01 g/cm^3 ($p=0.01$). However, there was no significant difference toward the apparent BMD when subjects having scoliosis ($p=0.530$).

3.4. Discussion

By using multiple regression analysis, cross-sectional measurements on

anthropometry showed that severe AIS girls had a significantly longer arm span and a slender figure, lower BMI when compared with the normal controls. This growth anomaly has been clinically observed in previous studies (Nordwall and Willner, 1975, Willner, 1974, Willner, 1975). In this study, a generalized low bone mass were also observed in the measurement of both femoral necks of the severe AIS patients by multiple regression analysis. This matches the previous findings of low BMD in AIS girls (Cheng *et al.*, 2006, Cheung *et al.*, 2006), and possibly being a prognostic factor to assess progression, since all our patients had severe AIS (Hung *et al.*, 2005).

In anthropometric analysis, the heights of the severe AIS girls and the normal control were compared. The measurement could be used to convert to corrected height by Bjure's formula (Bjure *et al.*, 1968). Similar study was reported to deduce different regression models in measuring the corrected height in AIS patients (Kono *et al.*, 2000). However, the difficulties in measurement of the corrected height have been discussed in particular for AIS patients with multiple curves (Kono *et al.*, 2000). Previous study showed positive linear correlation between arm span and standing height in healthy adolescents ($r^2 = 0.99$) (Cheng *et al.*, 1996). Hence, with this present study, due to the presence of different curves types in the AIS girls, it is more reliable and feasible to use arm span as a measure of body height. As there was minimal effect from the spinal deformity toward arm span, this can minimize the inaccurate prediction of the height using the conventional Bjure's formula (Bjure *et al.*, 1968) with only the major Cobb's angle. By multiple regression analysis, it was shown that age increment would significantly increase the arm span by 0.58 cm, with a standard error of 0.18 cm, in a healthy individual (Table 3.2). However, for AIS

patient, the arm span would be significantly longer than the normal controls by 2.56 cm with a standard error of 0.75 cm. This finding was similar to previous reports (Cheng *et al.*, 2000, Cheung *et al.*, 2006, Lee *et al.*, 2005a, Siu King Cheung *et al.*, 2003) on AIS girls having a longer arm span than normal controls. In Lee *et al*'s study (2005b), they compared the anthropometry and BMD of 619 AIS girls, of different severities, with 300 healthy controls aged 11-16 years old. It reported that the arm span and leg length among the moderate and severe AIS girls were longer than the controls. After adjusting for the age, the arm span and leg length was significantly correlated with curve severity ($p=0.015$). Thus, the observed longer arm span could reflect the presence of abnormal bone growth in AIS girls with a large curvature.

For the body weight and body mass index, there are mixed results showing that AIS patients were similar in weight (Cheng and Guo, 1997, Vetter *et al.*, 1991) or leaner than normal healthy individuals (Akhtar *et al.*, 2005, Felsenberg and Boonen, 2005). In the present study, the severe AIS girls had a lower BMI than the normal controls (Table 3.2). By multiple regression analysis, the severe AIS patients had a significantly lower BMI ($p<0.001$) than the normal adolescent girls by 1.43 kg/m^2 with a standard error of 0.22 kg/m^2. Nevertheless, the present findings agrees with previous literatures (Shohat *et al.*, 1988, Willner, 1975) that severe AIS girls are leaner and likely to be below the normal range of BMI.

The abnormal growth, demonstrated in the arm span and BMI, might be a reflection of disproportional endochondral ossification in the growth plates of long bone and endplate in vertebral bodies (Juul, 2001, Smith *et al.*, 1994). In Guo *et al.* (2003)

MRI study, the anterior vertebral bodies of AIS girls were found to be taller and leaner when compared with age-matched controls. It also demonstrated the relative overgrowth of the anterior column of the spine. The authors hypothesized that the abnormality was due to the disproportionate growth between endochondral ossification and membranous ossification. The findings coupling with the clinical observation that the severe AIS girls are slender and leaner than the normal adolescent girls points to a systemic disorder in skeletal growth.

For the BMD measurements, the severe AIS girls were grouped according to the site and convexity of major curve (Table 3.3). BMD Measurement showed lower BMD in bilateral sides of the femoral necks of the severe AIS girls when compared with the healthy controls. The results of multiple regression analysis showed that the BMD of the bilateral femoral necks was significantly related to age, body weight and curve severity in the AIS girls. In the severe AIS girls, the BMD on the convex side of the femoral neck have a lower mean value than the concave side when compared with the normal control. This finding was similar to Hans *et al.* (1996), through dual-photon x-ray absorptiometry measurments, this study measured the femoral BMD to determine whether there is a difference between left and right side and its relationship to scoliosis convexity. The result showed that the BMD at the femoral neck was lower on the convex side in fifteen female patients with structural lumbar scoliosis. Another study on subjects with adult lumbar scoliosis (Rumancik *et al.*, 2005) showed a higher BMD on the concave side of the femoral neck than the convex side by DXA measurements. This occurrence was postulated to be due to the greater mechanical loading within the osteoporotic bone, less bone formed and higher bone loss on the convex side. However, a study pointed out multiple factors

could affect these results, including rotation of the spine, compression of the vertebrae and biomechanical adaptation within the cancellous or cortical bone tissue (Routh *et al.*, 2005). The current study showed significantly lower BMD in both femoral necks of the AIS girls than the normal controls. This result agreed with the previous reports on the high prevalence of low BMD in a mixed population of AIS girls with different severities (Hung *et al.*, 2005, Lee *et al.*, 2003).

The measured apparent BMD is the mass of mineral in a skeletal region, where not all of which is bone. These regions are composed of mineral fashioned into cortical bone and trabecular bone within a central medullary cavity containing marrow. The BMDs of the cortical and apparent midshafts of radius were measured by pQCT (Table 3.4). As this study is focused on the mid-shaft BMD undergoing membranous ossification, only the BMD is taken into account, which assumed osteoblasts bone formation without cartilagenesis. Thus, the parameters of BMC and cross-sectional area (CSA) were not considered. In order to determine whether there is a difference due to maturity, the subjects were divided into 2 groups based on the age of 16 years old. By using multiple regression models, difference between the severe AIS and normal controls were only found after skeletal maturity, an observation that could be possibly explained by the more rapid acquisition in BMD in the normal controls with its accumulated effects. This observation has no apparent contradiction to previous reports on the presence of systemic low BMD in AIS girls (Cheng *et al.*, 2000, Cheung *et al.*, 2006). For their studies reported findings on systemic low BMD in skeletally-matured AIS girls, but not involving immatured AIS girls. However, the difference between the maturations may be due to the intrinsic difficulties in accurately estimating the cortical BMD of the midshaft

of radius in the younger severe AIS subjects. Previous report (Cooper *et al.*, 2007) showed that porosity of the cortical bone might be taken into account when measuring the material density. As in the current technology, there is no available method to depict the porosity of these pores *in vivo*, unless the tissue is taken from the subject for histology or microCT evaluations (which will form part of another series of ongoing studies in the same center).

These results revealed poor BMD may reflect the presence of poor bone mechanics in AIS patients. A recent study (Cheng *et al.*, 2006) revealed that over 86% of osteopenic AIS patients had persistently low BMD, at both distal tibia and femoral neck regions, at the time of skeletal maturity. The observed osteopenia in AIS girls may indicate a strong likelihood of developing osteoporosis at adulthood (1993). Osteoporosis is now defined as the micro-architectural deterioration of the bone with associated lost in bone mechanical strength. The bone strength is governed not only by the micro-architecture, but also the material property (Felsenberg and Boonen, 2005). The material properties of properly mineralized bone are contributed by a combination of stiffness and brittleness, while weakly mineralized bone are mainly from brittle fragments (Currey, 1984, Turner, 2002, Zioupos *et al.*, 2000). Mechanical property accounts for over 60% of the bone density observed (Bouxsein, 2003, Carter and Hayes, 1977, Rice *et al.*, 1988). In osteoporosis of the elderly, changes in geometry and material properties are the main causes for the loss of bone strength (Mosekilde, 1998).

In previous studies, Cheng *et al.* (1997) reported that the presence of systemically low BMD in AIS patients was independent to severity or curve patterns. Based on

our results, the severe AIS girls demonstrated a similar phenomenon of having low BMD like those reported of generalized AIS population (Cheng *et al.*, 2000, Cook *et al.*, 1987b, Thomas *et al.*, 1992). Study on histology and histomorphometry of cancellous bone from AIS patients (Cheng *et al.*, 2001) also showed lower osteocytes count in the trabecular bone characterized with smooth and continuous borders. This suggested a possible disturbance of bone remodeling in AIS patients. Bone remodeling is the dynamic interaction of activities of osteoblasts and osteoclasts. This consists of three main steps: 1) production of extracellular organic matrix (osteoid), 2) mineralization of the matrix to form bone and 3) bone remodeling by resorption and reformation. Various factors can affect the activity of both bone-forming osteoblasts and bone-resorping osteoclasts by modulating the synthesis (signaling) of growth factors and cytokines (Cai *et al.*, 2008, Galliera *et al.*, 2008, Korf-Klingebiel *et al.*, 2008).

Recent studies suggested that melatonin has an effect on bone remodeling (Ladizesky *et al.*, 2003, Ladizesky *et al.*, 2001). An *in vitro* study showed that melatonin increased the proliferation in normal human bone cells and osteoblastic cells to increase gene expression of sialoprotein and other bone marker proteins, like alkaline phosphatase and osteocalcin in bone cells (Roth *et al.*, 1999), which may promote matrix mineralization. Hence, it is logical to speculate that melatonin signaling dysfunction and/or abnormal cellular responses to melatonin might account for the low BMD and abnormal bone growth observed in AIS patients.

Table 3.1. Age distributions of subjects recruited in the current study

Age (yr)	Severe AIS Subjects (n=224)		Normal Controls (n=160)	
	No. of Patients	% of Distribution	No. of Patients	% of Distribution
13	21	9.4 %	16	10.0 %
14	29	12.9 %	12	7.5 %
15	25	11.2 %	19	11.9 %
16	45	20.1 %	17	10.6 %
17	45	20.1 %	23	14.4 %
18	20	8.9 %	32	20.0 %
19	28	12.5 %	23	14.4 %
20	11	4.9 %	18	11.3 %

Table 3.2. Multiple regression analysis on the arm span and body mass index between severe AIS patients and control subjects

		Arm span (cm)	BMI (kg/m^2)
	R	0.223	0.339
	R squared	0.05	0.115
	Regression sig (p value)	<0.001	<0.001
Constant	Beta	148.232	17.99
	S.E.	3.057	0.911
	P value	0.0001	0.045
Variables			
Age	Beta	0.580	0.107
	S.E.	0.179	0.053
	P value	0.001	0.045
Severity	Beta	2.566	-1.434
	S.E.	0.748	0.223
	P value	0.001	<0.001

Table 3.3. Multiple regression analysis on the BMD of the bilateral femoral neck in severe AIS patients and control subjects.

		Non-Dominant FN BMD (g/cm^2)	Dominant FN BMD (g/cm^2)	Convex side FN BMD (g/cm^2)	Concave side FN BMD (g/cm^2)
	R	0.535	0.534	0.537	0.526
	R squared	0.286	0.285	0.288	0.277
	Regression (p value)	<0.001	<0.001	<0.001	<0.001
Constant	Beta	0.383	0.387	0.381	0.394
	S.E.	0.049	0.049	0.049	0.049
	P value	<0.001	<0.001	< 0.001	< 0.001
Variables					
Age	Beta	0.007	0.007	0.007	0.007
	S.E.	0.002	0.002	0.002	0.002
	P value	0.005	0.003	0.004	0.006
BW	Beta	0.007	0.007	0.007	0.007
	S.E.	0.001	0.001	0.001	0.001
	P value	< 0.001	<0.001	< 0.001	< 0.001
Severity	Beta	-0.035	-0.035	-0.036	-0.033
	S.E.	0.01	0.01	0.01	0.01
	P value	<0.001	<0.001	<0.001	<0.001

FN=Femoral neck; BMD=Bone mineral density

Table 3.4. Multiple regression analysis on the BMD on the midshaft of radius in severe AIS patients and control subjects under 16 years old.

Age < 16 years old		Cortical BMD (g/cm^3)	Apparent BMD (g/cm^3)
	R	0.427	0.126
	R squared	0.182	0.016
	Regression (p-value)	<0.001	0.601
Constant	Beta	1.238	0.874
	S.E.	0.067	0.088
	P value	<0.001	<0.001
Variables			
Age	Beta	0.024	0.009
	S.E.	0.005	0.007
	P value	<0.001	0.185
Severity	Beta	0.000	0.003
	S.E.	0.009	0.011
	P value	0.948	0.794

BMD=Bone mineral density

Table 3.5. Multiple regression analysis on the BMD on the midshaft of radius in severe AIS patients and control subjects over 16 years old.

Age > 16 years old		Cortical BMD (g/cm^3)	Apparent BMD (g/cm^3)
	R	0.365	0.195
	R squared	0.133	0.038
	Regression (p value)	<0.001	0.019
Constant	Beta	1.464	0.941
	S.E.	0.034	0.053
	P value	<0.001	<0.001
Variables			
Age	Beta	0.010	0.006
	S.E.	0.002	0.002
	P value	<0.001	0.010
Severity	Beta	-0.010	-0.004
	S.E.	0.005	0.007
	P value	0.050	0.530

BMD=Bone mineral density

Chapter 4 Response of Osteoblasts to Melatonin in AIS Girls - *In vitro* Study

4.1. Introduction

In the study presented in detail in Chapter 3, severe AIS girls had been shown to have low BMD and abnormal anthropometric parameters including lower BMI and longer arm span when compared with normal controls. The results pointed strongly to abnormal peri-pubertal bone growth and bone remodeling which matched with a number of other related studies (Cheung *et al.*, 2006, Willner, 1975).

Normal bone matrix consists of a unique composite of living cells embedded within a three dimensional, mineralized, honeycomb like structure. The bone remodeling process is carried out by three types of cells; osteoblasts, osteoclasts and osteocytes. Osteoblasts are responsible for the synthesis of bone matrix, while osteoclasts are responsible for the resorption of mineralized bone. Osteocytes are osteoblasts entrapped within the lacunae. In order to balance bone formation and resorption in healthy individuals, osteoblasts secrete factors that regulate the differentiation of osteoclasts. Osteocytes are responsible for secreting factors regulating the activity of both osteoblasts (Hartmann, 2006) and osteoclasts (Seeman, 2006). As part of the normal bone remodeling process, bone is constantly reabsorbed by osteoclasts and replaced by osteoblasts (Ducy, 2000). These interactions are essential for maintaining a balance between the rates of bone loss and gain. Disruption in the regulated networks can result in rapid bone loss (i.e. osteoporosis) together with fragility fractures (Boskey and Posner, 1984, Felsenberg and Boonen, 2005, Parfitt, 1984).

It has been reported that melatonin plays a vital role in preventing bone loss in

osteoporosis of animal models (Ladizesky *et al.*, 2003, Ladizesky *et al.*, 2001). Ladizesky *et al.* (2003) reported that daily intake of melatonin might prevent the bone loss in ovariectomized rats. Other clinical studies have shown that aging would decrease melatonin secretion, eventually increasing the risk to develop osteoporosis (Sandyk *et al.*, 1992, Witt-Enderby *et al.*, 2006). In addition, previous reports have demonstrated that melatonin enhances proliferation (Nakade *et al.*, 1999, Satomura *et al.*, 2007) and differentiation (Roth *et al.*, 1999, Satomura *et al.*, 2007) of bone cells. Satomura *et al.* (2007) showed a dose-dependent response in enhancing proliferation, differentiation in human osteoblasts and mineralization of bone matrix.

Studies on the serum melatonin concentrations in AIS girls have been reported (Bagnall *et al.*, 1996, Brodner *et al.*, 2000, Fagan *et al.*, 1998, Hilibrand *et al.*, 1996, Machida *et al.*, 1996). However, controversies on whether there are differences on the melatonin serum concentration between AIS girls, of different severities, and healthy controls still prevail. Machida *et al.* (1996) had shown that AIS patients with progressive curves have significantly lower melatonin serum concentrations than the controls. Recently, Moreau *et al.* (2004) found dysfunction of melatonin signaling pathway in osteoblasts derived from AIS patients. The osteoblasts from AIS patients responded differently to melatonin's inhibitory effect on the forskolin-stimulated cyclic AMP. It showed some of the AIS osteoblasts failing to inhibit the forskolin-stimulated cyclic AMP, upon the addition of melatonin. The AIS patients were subdivided into three groups according to their degree of cellular responses toward melatonin on the accumulation of cyclic AMP concentration (Moreau *et al.*, 2004). However, the functional characteristic of individual groups

has yet to be determined. Moreover from previous genetic association study by Qiu *et al.* (2007a), melatonin 1B receptor polymorphism was shown to be associated with the occurrence of AIS.

It was hypothesized that there could be abnormal cellular responses of osteoblasts from AIS patients to melatonin physiologically. Hence, the objective of the study described in this chapter is to determine the effect of melatonin on proliferation and differentiation of osteoblasts isolated from AIS patients and to compare the response with normal controls.

4.2. Methodology

4.2.1. Subjects Recruitments

From the series of severe AIS patients recruited for the previous study described in Chapter 3, only a few subjects were recruited due to the time limitation and difficulties in obtaining consent from the patients and their parents. Consents to take intraoperative bone biopsy were obtained from 13 patients and their parents who subsequently underwent corrective spinal surgery and instrumentation within the period of this thesis study (Table 4.2). The inclusion and exclusion criteria and the clinical diagnosis of AIS were described in Chapter 3.

Intraoperative bone biopsies were also obtained from 9 normal control subjects, aged between 14 to 28 who were undergoing reconstructive surgery for trauma-related condition, from the same hospital during the same period (Table 4.3). The absence

of scoliosis, spinal problem and other bone metabolic diseases were confirmed by an experienced orthopedic surgeon preoperatively.

The protocol was approved by the Clinical Research Ethics Committee of the University (Appendix II). An informed consent was given in details to all the subjects and their parents prior to their recruitment into the study (Appendix III-VI).

4.2.2. Cell Isolation

4.2.2.1. Bone Biopsies for Osteoblasts Isolation

Bone biopsies of iliac crest or facet joint were obtained intraoperatively from the AIS girls (Figure 4.1). For the osteoblasts from normal controls, bones biopsies were obtained from normal bone of the distal femur during surgery, or unaffected areas for the tumor cases. The study protocol was approved by the Clinical Research Ethics Committee of the University (Appendix II). Informed consents were obtained from all the subjects and their parents prior to collecting any samples for the study (Appendix III-VI).

4.2.2.2. Materials and Reagents

Phosphate Buffered Saline (PBS)

The following 1.44 g Na_2HPO_4 (Merck & Co., Darmstadt, Germany), 0.24 g KH_2PO_4 (Merck & Co., Darmstadt, Germany), 8.0 g NaCl (Merck & Co., Darmstadt, Germany) and 0.2 g KCl (Merck & Co., Darmstadt, Germany) were dissolved in 900 ml distilled water. The pH was adjusted to 7.4 and the final volume was made up to

1000 ml with distilled water. Sterilize the solution by autoclave and store at 4°C.

Alpha-Minimal Essential Medium (α-MEM)

One pack of αMEM (Invitrogen, Carlsbad, USA), 2.2 g $NaHCO_3$ (Riedel-de Haen, Seelze, Germany) and 0.1 g D-valine (Sigma, St.Louis, USA) were dissolved in 800 ml distilled water. The pH adjusted to 7.4 and the final volume was made up to 1000 ml with distilled water. The solution was passed through a bottle top filter.

Osteogenic Medium

Fetal bovine serum (FBS) (10 ml) (Gibco-Invitrogen, Carlsbad, USA) and penicillin/neomycin/streptomycin antibiotic cocktail (PNS) (1.0 ml) (Invitrogen, Carlsbad, USA) in 89 ml α-MEM were mixed gently to prevent foam formation. Then, 1.0 ml 10 mM ascorbic acid (Sigma, St.Louis, USA), 1.0 ml 10 mM β-glycophosphate (Sigma, St.Louis, USA) and 0.1 ml 10^{-7} M dexamethasone (Sigma, St.Louis, USA) were added to the solution. The solution was mixed gently and stored at 4°C in the dark.

ALPase Buffer

The following including 10.2 g $MgCl_2.6H_2O$ (Panreac, Barcelona, Spain), 12.1 g Tris-HCl (Sigma, St.Louis, USA), 5.8 g NaCl (Amresco, Solon, USA) and 1.0 ml 0.1% Tween-20 (Amresco, Solon, USA) were dissolved in 1000 ml distilled water. The solution was mixed gently.

ALPase Substrate Buffer

The solution was freshly prepared by adding 25 µl 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega, Madison, USA) and 50 µl nitro blue tetrazolium (NBT) (Promega, Madison, USA) into 4.9 ml ALP buffer. The solution was mixed gently and stored in the dark.

4.2.2.3. Isolation of Osteoblasts from Bone Biopsies

Bone fragments were first rinsed with plain α-MEM supplemented with 10% PNS to prevent possible bacterial contamination. The trabecular bones were cut and minced into small pieces with a sharp bone cutter in sterile conditions. Excess blood was removed by gentle rinsing in sterile PBS. The fragments were then plated onto a 6-well culture plate and incubated for 28 days in basal culture medium of α-MEM (Invitrogen, Carlsbad, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, USA) and 1% PNS (Invitrogen, Carlsbad, USA). The culture was maintained at 37°C in a humidified atmosphere of 5% carbon dioxide (CO_2). The medium was renewed every 3-4 days. When the osteoblasts reached 80 – 90% confluence, the cells were harvested for assessment. A clear illustration of the isolation process is shown in Figure 4.1. In confirming the osteoblastic characteristic, cells were detected for the presence of ALPase, a bone forming marker. In addition, cells passage for more than 3 times were excluded from this study.

4.2.2.4. Alkaline Phosphatase Enzyme Cytochemistry

To confirm the osteoblastic characteristic of the isolated cells, their capacity to produce ALP was assessed. In brief, the cells were seeded at a density of 7500 cells per well in a 6-well plate, and cultured in the presence of osteogenic medium for 3 days. Thereafter, the cells were fixed in 70% alcohol and washed with ALPase buffer. Then, the cells were incubated at 37°C with ALPase substrate buffer for 45 minutes. The reaction was stopped by adding distilled water. Positive purple signal was evaluated under microscope.

4.2.3. Effect of Melatonin on Proliferation and Differentiation of AIS Osteoblasts

4.2.3.1. Materials and Reagents

Dulbecco's Modified Eagle Medium (DMEM)

One pack of DMEM (Invitrogen, Carlsbad, USA), 3.7 g $NaHCO_3$ (Riedel-de Haen, Seelze, Germany) and 0.1 g D-valine (Sigma, St.Louis, USA) were dissolved in 800ml distilled water. The pH adjusted to 7.4 and the final volume made up to 1000 ml with distilled water. The solution was passed through a bottle top filter and stored at 4°C.

Melatonin

Melatonin (23.23 mg) (Sigma, St.Louis, USA) was dissolved in 1 ml DMSO (Sigma, St.Louis, USA) in the dark and mixed gently. The concentration of the prepared solution is 0.1M. Serial dilutions were performed to obtain other concentrations.

When added into the medium, the final DMSO concentration should not exceed 0.1%.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Stock Solution

MTT powder (0.01 g) (Sigma, St.Louis, USA) was dissolved in 2.0 ml autoclaved PBS. Stock solution was sterilized by filtering through a 0.22-μm membrane (Millipore, Bedford, USA). The solution was kept away from light and stored at -20°C.

MTT Working Solution

The solution was freshly prepared by mixing 1.0 ml MTT stock solution in 9.0 ml DMEM (Invitrogen, Carlsbad, USA). The solution was mixed gently and kept away from light.

Diethanolamine (DEA) Buffer

The followings including 0.2 g $MgCl_2.6H_2O$ (Merck & Co., Darmstadt, Germany), 0.5 ml 4M HCl (Merck & Co., Darmstadt, Germany) and 0.5 ml diethanolamine (Sigma, St.Louis, USA) were mixed in 99 ml distilled water. The solution was mixed gently and stored at room temperature.

ALPase Working Reagents

Approximately 25 ml of 2-amino-2-methyl-1-propanol (Reagent A) was mixed in 50 ml of 4-nitrophenylphosphate (Reagent B). The solution was mixed gently and stored at 4°C for a shelf-life of 2 months. Prior to use, the solution was allowed to

pre-warm at 37°C for at least 30 minutes.

4.2.3.2. Cell Viability Assay

The effect of melatonin on proliferation of the normal human and AIS osteoblasts was analyzed by using MTT cell viability assay to measure the total number of viable cells before and after 3 days of melatonin treatment, as described by Satomura *et al.* (2007). The assay is dependent on cellular reduction of MTT by mitochondrial dehydrogenase present in viable cells to form a red formazan product representing the proliferative activity of the cells (Mosmann, 1983). In brief, the cells were seeded at a density of 3000 cells per well in 96-well culture plates and cultured in α-MEM containing 1% FBS and various concentrations (10^{-9}, 10^{-5} and 10^{-4} M) of melatonin for 3 consecutive days, with daily renewal of medium and melatonin. At the end of 3 days, the medium was removed and rinsed with sterile PBS. The MTT solution was added and incubated for 4 hour at 37°C. Thereafter, the MTT solution was carefully removed. The formazan in the cells was released with the addition of DMSO and shaken gently at room temperature. The absorbance of the amount of formazan formed was measured at 570 nm using a microplate reader (Gemini, Osaka, Japan). All measurements were performed in quadruplicate.

4.2.3.3. Alkaline Phosphatase Activity

The ALPase enzyme activity was determined using p-nitrophenyl phosphate (PNPP) as the substrate (Lowry *et al.*, 1954). In brief, cells were plated at a density of 7500

cells per well in a 96-well culture plate. The cells were cultured in α-MEM containing 10% FBS until confluence. Thereafter, the cells were treated overnight with osteogenic medium to induce the ALPase activity of the cells. Afterwards, daily treatments with various concentrations (10^{-9}, 10^{-5} and 10^{-4} M) of melatonin in α-MEM containing 1% FBS was added for 3 consecutive days. At the end of 3 days, the medium was removed and rinsed with sterile PBS. The cells were then lysed with DEA buffer at room temperature. To determine the ALPase activity, the pre-warmed PNPP substrate from a commercial kit (Biosystems, Barcelona, Spain) was added. The reaction mixture was mixed gently for 5 minutes at room temperature. The absorbance due to ALPase activity was measured at 405 nm using a microplate reader (Gemini, Osaka, Japan). The level of ALPase activity was normalized by the total number of viable cells, determined by MTT assay. All measurements were performed in quadruplicate.

4.2.4. Data Analysis

All data were expressed as mean ± S.D. This was taken from the average of the cell cultures performed in either triplicate or quadruplicate. Statistical significance of the difference from the vehicle controls was analyzed with a two-tailed Student's t-test. Statistical significance on the difference between the groups in the same melatonin concentrations was determined by one-way analysis of variance (ANOVA). The alpha value was set to 0.05 to be considered statistically significant. SPSS software (version 11; SPSS, Inc., Chicago, IL) was used for statistical analysis.

4.3. Results

4.3.1. Isolated Osteoblasts from Normal Human and AIS Patients

The isolated osteoblasts, from normal human (Figure 4.2) and AIS patients (Figure 4.3), were fibroblast-like in morphology (Figure 4.2A and Figure 4.3A) and exhibited positive ALPase activity (Figure 4.2B and Figure 4.3B).

4.3.2. Effect of Melatonin on Osteoblasts Proliferation

In Figure 4.4, the proliferation of the normal human osteoblasts was enhanced in a dose-dependent manner when the cells were exposed to a serial concentration of melatonin for 3 days. As measured by MTT assay, the percentage of proliferation in normal human osteoblasts was enhanced by 6%, 11% and 12% with the serial concentration of melatonin (10^{-9}, 10^{-5} and 10^{-4} M) when compared with the vehicle, respectively. The enhancement was significant at both physiological (10^{-9} M) ($p=0.021$) and pharmacological dosages of melatonin (10^{-5} M) ($p=0.005$), (10^{-4} M) ($p=0.004$). However, there was no significant overall difference on the proliferation of the AIS osteoblasts to melatonin ($p>0.05$) in comparison with the vehicle. In comparing the normal controls with the AIS osteoblasts, the differences in proliferation at different melatonin concentrations were statistically significant at 10^{-9} M ($p=0.014$), 10^{-5} M ($p=0.008$) and 10^{-4} M ($p=0.002$).

The osteoblasts isolated from the AIS patients were divided into two groups basing on their response to the physiological dose (10^{-9} M) of melatonin (Figure 4.6). In the first group (Group A, n=3; Figure 4.7), melatonin treatment enhanced the

proliferationof osteoblasts, at a trend similar to the normal control, at all concentrations (10^{-9}, 10^{-5} and 10^{-4} M). The enhancement on the proliferation of osteoblasts was statistically significant at 10^{-5} M ($p=0.021$) when compared with the vehicle. However, this enhancement at melatonin concentrations 10^{-9} M ($p=0.109$) and 10^{-4} M ($p=0.263$) was not statistically significant. The second group (Group B, n=10; Figure 4.7) showed an inhibitory trend on the proliferation of osteoblasts by increasing concentrations of melatonin. The inhibition was statistically significant at all concentrations of 10^{-9} ($p=0.006$), 10^{-5} ($p=0.016$) and 10^{-4} M ($p=0.006$) when compared with the vehicle.

When compared between the normal controls and Group B (Figure 4.7), there was also statistical significant differences in the number of osteoblasts at all melatonin concentrations ($p<0.001$). In the comparison between Group A and Group B, there were significant difference on the effect of melatonin toward the number of osteoblasts concentrations of 10^{-9} ($p=0.031$) and 10^{-5} ($p<0.001$) without an associated difference at the melatonin concentrations of 10^{-4} ($p=0.109$), which could be related to the large standard deviations in Group A.

4.3.3. Effect of Melatonin on Cell Differentiation

The rate of ALPase activity was measured as an indicator for the degree of osteoblast differentiation, with normalization by MTT cell viability assay. Figure 4.5 shows that the ALPase activity of normal human osteoblasts was enhanced in a dose-dependent manner when the cells were exposed to a serial concentration of melatonin for 3 days. The percentage of ALPase activity was enhanced by 8%,

22% and 32% with the serial concentration of melatonin (10^{-9}, 10^{-5} and 10^{-4} M), respectively. The enhancement was significant ($p=0.036$) at the highest pharmacological dosage of melatonin (10^{-4} M) and not at the melatonin concentrations of 10^{-9} M ($p=0.103$) and 10^{-5} M ($p=0.083$) when compared with the vehicle. However, there was no significant difference on the ALPase activity of the AIS osteoblasts to melatonin ($p>0.05$) in comparing with the vehicle. In comparing the normal controls with the AIS osteoblasts, there was no significant difference in ALPase activity following the addition of melatonin ($p>0.05$).

Based on classification from cell proliferation study, the osteoblasts isolated from the AIS patients were similarly grouped into Group A and B and tested for their cellular differentiation in response to various concentrations of melatonin (Figure 4.8). In Group A (Figure 4.8), melatonin treatment promoted ALPase activity, similar to the normal control, at all concentrations (10^{-9}, 10^{-5} and 10^{-4} M) when compared with the vehicle. However, the promotion was not statistically significant at any of the melatonin concentrations ($p>0.05$). While for Group B (Figure 4.8), it showed an inhibition on the ALPase activity toward melatonin concentrations of 10^{-9} and 10^{-4} M when compared with the vehicle. While at the melatonin concentration of 10^{-5} M, it showed the promotion of ALPase activity. However, the enhancement of ALPase activity might be affected by the large standard deviation observed in this melatonin concentration (10^{-5} M). None of the ALPase activity response in Group B was statistically significant toward melatonin ($p>0.05$).

In comparing the normal controls with Group B (Figure 4.8), there were significant differences in ALPase activity at the highest melatonin concentrations ($p=0.042$)

(10^{-4}), but not at the other melatonin concentrations 10^{-5} M ($p=0.079$) and 10^{-5} M ($p=0.505$). No significant difference between the ALPase activity was found between Group A and normal controls ($p>0.05$). In comparing between Group A and Group B, there was no significant difference in the ALPase activity following the addition of melatonin ($p>0.05$).

4.4. Discussion

In this study, melatonin dose-dependently enhanced proliferation and differentiation of normal human osteoblasts (Figure 4.4 and 4.5). However, there was the lack of enhancement effects on proliferation and differentiation in the AIS osteoblasts to melatonin (Figure 4.4 and 4.5). When compared between the normal controls with the AIS osteoblasts, there was significant difference in all concentrations of melatonin toward proliferation ($p<0.05$). Hence, the study further classified the AIS osteoblasts into two groups based on the proliferative response toward the physiological dose of melatonin (Figure 4.6). Based on this classification, osteoblasts from patients with AIS showed heterogeneous response toward melatonin on proliferation and differentiation (Figure 4.7 and Figure 4.8). One of the groups (Group A) showed enhancement effect of melatonin, while the other group (Group B) showed inhibitory effect. Group A behaved similarly to the proliferative trend of normal osteoblasts toward melatonin, while Group B showed inhibitory response toward melatonin. Significant difference in cellular proliferation was found between the normal human osteoblasts and Group B AIS at physiological and pharmacological concentrations of melatonin ($p<0.001$) and also between Group A and Group B at the physiological melatonin concentrations ($p=0.031$). Similar

differences were not found when comparing the normal controls with Group A AIS. This provides reasonable grounds supporting the functional classification of AIS patients into 2 groups of A and B. As proliferation declines before the onset of differentiation, it is likely that the abnormal response to melatonin could be seen in cellular proliferation first. Using this classification, statistical significance on the ALPase activity ($p=0.042$) was also found between the controls and Group B at the highest pharmacological concentrations of melatonin (10^{-4} M).

On the effect of melatonin on cellular proliferation, there were three studies mainly demonstrated the different effects on bone cells (Nakade *et al.*, 1999, Roth *et al.*, 1999, Satomura *et al.*, 2007). In the rodent's cell line, MC3T3-E1 and ROS 17/2.8, melatonin showed minimal suppression of cell proliferation (Roth *et al.*, 1999). Nakade *et al.* (1999) reported that melatonin stimulated proliferation of primary human osteoblasts. Similarly, Satomura *et al.* (2007) showed that melatonin at pharmacological doses could enhance proliferation in primary human osteoblasts as well. The slight difference in reports might be due to species variation, where the latter two reports used human osteoblasts instead of rodent cells.

In this study, melatonin showed a similar dose-dependent stimulatory effect on the normal human osteoblasts (Figure 4.4). While in the AIS osteoblasts, there was no significant enhancement on proliferation to melatonin (Figure 4.4). However, when it was compared between normal human osteoblasts and the AIS osteoblasts, there were significant differences at all concentrations of melatonin. On the contrary, determination of the cell viability in the presence of melatonin allowed a functional classification of patients with AIS into 2 groups based on their responsiveness

toward the physiological concentration of melatonin (Figure 4.6). In Figure 4.7, Group A showed the enhancement in proliferation with the addition of melatonin, similar to the trend of normal controls. While the other group, Group B demonstrated inhibitory response in viable osteoblast counts with the addition of melatonin. The response in Group A was similar to that of normal control. This might indicate that the etiology of this group of patients might not be affected by melatonin pathway dysfunction. In current studies, melatonin showed inhibitory effect on proliferation to melatonin in osteosarcoma (Hill and Blask, 1988, Panzer, 1997, Toma *et al.*, 2007). However, the inhibitory response of melatonin in Group B is similar to other studies on suppressing osteoblast proliferation. It was shown that by blocking the ERK1/2 pathway inhibitor with antagonists (PD98059), melatonin can attenuated the ERK1/2 phosphorylation to significantly reduce the proliferation and differentiation in osteoblasts (Dai *et al.*, 2007). In addition, antagonist of estrogen receptor had shown to decrease cell proliferation by slowing down the osteoblasts maturation (Ogita *et al.*, 2008). Therefore, it is possible that these molecular mechanisms might also be involved in the proliferation of the primary osteoblasts from the selected group of AIS patients.

Based on the results, Group A demonstrated an enhancement effect by melatonin on both proliferation and differentiation, which was similar to the normal human osteoblasts. No significant difference was found between these two groups, indicating that the osteoblasts in Group A do not have abnormal response to melatonin. Unlike Group A, the osteoblasts in Group B showed a drop in proliferation and differentiation toward melatonin. As there was significant difference between Group B and Group A, this could imply that the osteoblasts from

Group B did have an abnormal response to melatonin. The different responses toward melatonin could imply the presence of heterogeneity in the AIS population.

The ALPase activity in the normal human osteoblasts showed a dose-dependent enhancement in response to increased concentrations of melatonin (Figure 4.5). This result is similar to the study reported by Satomura *et al.* (2007) on melatonin enhancing differentiation markers and ALPase activity in human osteoblasts. While in the osteoblasts from the generalized AIS patients, there was a lack of enhancement effect in the ALPase activity toward the addition of melatonin. Likewise, there was no significant difference in the ALPase activity between the normal human osteoblasts and AIS osteoblasts. This might be due to the complication involved in the process of differentiation, which other substances (e.g. estrogen) might be interact with melatonin (Radio *et al.*, 2006, Suzuki and Hattori, 2002). Hence, when the AIS osteoblasts were subgroup according to that of the proliferation assay, two groups of responsiveness were shown again (Figure 4.8). Group A showed promoted effect in ALPase activity with the addition of melatonin, similar to the trend of normal controls. In Group B, inhibitory effect on ALPase activity was found with the increasing concentrations of melatonin. In a study on human mesenchymal stems cell (Radio *et al.*, 2006), melatonin was found to enhance ALPase activity by 50% relative to osteogenic media alone. However, the melatonin-mediated increases in ALPase activity could be blocked by the presence of MT2 antagonists. In general, the promotion of osteoblast differentiation has been shown to be mediated through the mitogen activated protein kinase (MAPK) cascade (Fujita *et al.*, 2002, Nakade *et al.*, 1999, Suzuki *et al.*, 2002, Xiao *et al.*, 2000). The inputs leading to MAPK activation in osteoblastic cells can be mediated through the

cyclic AMP (Fujita *et al.*, 2002). Once activated, MAPK has been shown to phosphorylate osteoblast-specific proteins, including osteocalcin (Xiao *et al.*, 2000). MAPKs are well known to play an essential role in controlling cell proliferation and differentiation (Johnson and Vaillancourt, 1994, Seger and Krebs, 1995, Zhang and Liu, 2002). In addition, Suzuki *et al.* (2002) reported that activation of ERK would play an important role in osteoblast proliferation. Recent findings also revealed that activation of the MAPK p38 is required for osteoblast differentiation, and p38 activation induces the expression of differentiation markers, such as ALPase and mineral deposition (Gallea *et al.*, 2001, Guicheux *et al.*, 2003, Hu *et al.*, 2003, Kakita *et al.*, 2004, Sowa *et al.*, 2002). Clearly, the role of the MAPK pathway in melatonin-induced osteoblast differentiation might be an important hint toward the abnormal response in Group B toward melatonin.

Insight into the mechanisms by which melatonin promotes osteoblast proliferation and differentiation can be implicated partly from recent reports on the melatonin signaling pathways. Moreover for the promoting effect of differentiation in bone cells, Roth *et al.* (1999) demonstrated that the transmembrane receptor might initiate the actions of melatonin in osteoblasts via signaling through the Gi protein. As the putative orphan receptor is regulated by cyclic AMP (Baler *et al.*, 1996), the binding of melatonin to its transmembrane receptor will result in changes in the levels of activity of the nuclear transcription factor in altering the expression of bone sialoprotein and differentiation. As it is shown clearly in the study of Satomura *et al.* (2007), the inhibition of cyclic AMP could enhance osteoblasts to proliferate and differentiate under the same melatonin concentrations. Similar findings on lower cyclic AMP accumulation resulting in higher cell proliferation and differentiation of

other cell types (Mallat *et al.*, 1998, Tsuboi *et al.*, 1996, Watson, 1976) have been reported. Hence, these findings further suggested that the abnormality in Group B of the AIS osteoblasts might be related directly to the accumulation in cyclic AMP as reported in recently (Azeddine *et al.*, 2007, Moreau *et al.*, 2004).

Detail micro-architecture of normal bone show the presence of bone-forming osteoblasts and bone-resorping osteoclasts (Boskey and Posner, 1984, Felsenberg and Boonen, 2005, Parfitt, 1984). Bone resorption is much faster than bone formation. The area of bone can be resorbed in 2-3 weeks but it takes at least three months to rebuild it (Harada and Rodan, 2003). Osteoblasts have a very important role in creating and maintaining skeletal architecture and are responsible for the deposition of bone matrix and the regulation of osteoclasts activity (Fonseca *et al.*, 2005). Along the osteoblastic differentiate, they acquire the ability to secrete bone matrix (Gori *et al.*, 2000). Both osteoblast proliferation and differentiation are controlled by the cyclic AMP signaling pathway. In the previous studies, parathyroid hormone (PTH) modulates proliferation and differentiation of osteoblasts through the cyclic AMP-mediated pathway (Partridge *et al.*, 1994, Siddhanti and Quarles, 1994). Moreover, cyclic AMP functional response elements have been reported in promoter regions of osteoblast associated genes (Pearman *et al.*, 1996, Povinelli *et al.*, 1992, Towler and Rodan, 1995). In the mechanism of matrix mineralization, a series of events occurs as cells undergo differentiation (Stein *et al.*, 1996). Proliferation declines before the onset of differentiation, and various osteoblastic marker genes, involved in extracellular matrix development and mineralization, are expressed. These include collagen type I, expressed maximally during proliferation and declines progressively, while ALPase and matrix GLA protein (MGP) expression start low

and peak during the matrix development/maturation stage. Other bone markers, osteopontin and osteocalcin, expressed would increase drastically during matrix mineralization (Stein *et al.*, 1996). However, the complex process of matrix mineralization in osteoblasts still remains unclear. Much evidence points to control of formation and maturation of extracellular matrix, providing an environment that facilitates mineral deposition (Robey, 1996, Stein *et al.*, 1996). Based on our data, the AIS osteoblasts demonstrated two different responses toward melatonin. Though a small sample size, Group B was found to have a lower BMD than Group A, possibly a result of the inhibitory response to melatonin. Similar to Moreau *et al.* finding (2004), the different responses in osteoblasts from AIS patients toward melatonin would most likely be a primary cellular functional defect involved in AIS, rather than a consequential secondary response from the spinal deformity. This could imply that the melatonin signaling pathway dysfunction might be the source of problem in certain subgroup of AIS patients.

For ethical reason, bone biopsies from normal adolescent controls is often very difficult to obtain even more so for girls than boys. In the present study, the age of the control was slightly older than the AIS patients. As mentioned by few authors (Cardinali *et al.*, 2008, Satomura *et al.*, 2007), an increase in age could be associated with decrease in expression of melatonin receptors. Therefore, it is speculated that the difference might be even more significant if the controls were of similar age.

Another possible limitation of the present study is the sex difference between the normal control and the AIS subjects. Although there are two female osteoblast samples in the normal controls, the remaining seven osteoblast samples were from

male subjects. While for all the AIS osteoblasts, they were isolated from female patients only. To our knowledge, there might be a fluctuation in the levels of sex hormones affecting bone metabolism in the patients, such as estrogens. The levels of these hormones are highest during the pubertal stage, especially in females. However, this study was conducted *in vitro* that was under a controlled culture environment without the influence of sex hormones to the osteoblasts from male and female. Hence, in general, the hormone fluctuation between genders would be minimal in this cellular study. Other studies have also demonstrated a similar effect of melatonin on human osteoblasts from samples of male and female (Azeddine *et al.*, 2007, Moreau *et al.*, 2004, Satomura *et al.*, 2007). Therefore, it is reasonable to have the comparison between the present normal controls and AIS patients.

In this study, it is clearly demonstrated the abnormal response of AIS osteoblasts to melatonin. Most importantly, there was heterogeneous response of osteoblasts from AIS patients to proliferation and differentiation when melatonin is added. One of the groups (group A) demonstrated a promoted response toward proliferation and differentiation with the addition of melatonin. While the other group (group B), it showed inhibition in proliferation and differentiation with the addition of melatonin. From previous studies, the polymorphism of MT2 to the occurrence of AIS might be a hint toward the abnormality between the two groups of AIS osteoblasts. Thus, it would be highly speculated that there might be related to the melatonin receptors in these AIS osteoblasts.

Table 4.1. Clinical data of patients with AIS

Case no.	Diagnosis	Curve Pattern	Gender	Age at Surgery (yrs)	Cobb Angle (°)	Risser Sign	Family History (Yes/No)
A1	AIS	R/L Double curve	F	13.8	80-46	2	Yes
A2	AIS	R/L Double curve	F	17.9	53-58	4	No
A3	AIS	R/L Double curve	F	13.8	90-63	4	No
A4	AIS	R/L Double curve	F	15.0	56-31	4	No
A5	AIS	R/L Double curve	F	12.6	31-64	2	No
A6	AIS	L/R/L Triple curve	F	16.2	37-67-42	5	No
A7	AIS	R/L Double curve	F	20.2	66-70	5	Yes
A8	AIS	R/L Double curve	F	14.6	36-75	1	Yes
A9	AIS	R/L Double curve	F	14.6	64-60	4	No
A10	AIS	L/R/L Triple curve	F	14.8	33-77-66	3	No
A11	AIS	R thoracic curve	F	18.7	65	5	No
A12	AIS	R/L Double curve	F	17.2	31-54	5	Yes
A13	AIS	L/R/L Triple curve	F	14.1	30-72-60	3	No

AIS = adolescent idiopathic scoliosis; R = right; L = left.

Table 4.2. Clinical data of control patients

Case No.	Type of Surgery where intraoperative bone biopsies were obtained	Curve Pattern	Gender	Age at Surgery (yrs)	Family History (Yes/No)
N1	ACL Reconstruction	Nil	M	22.8	NA
N2	Musculoskeletal Tumor Surgery	Nil	F	19.5	NA
N3	ACL Reconstruction	Nil	M	25.8	NA
N4	ACL Reconstruction	Nil	M	27.4	NA
N5	ACL Reconstruction	Nil	F	20.2	NA
N6	ACL Reconstruction	Nil	M	23.2	NA
N7	ACL Reconstruction	Nil	M	28.1	NA
N8	ACL Reconstruction	Nil	M	19.0	NA
N9	Musculoskeletal Tumor Surgery	Nil	M	14.8	NA

ACL = Anterior cruciate ligaments; NA = Not applicable

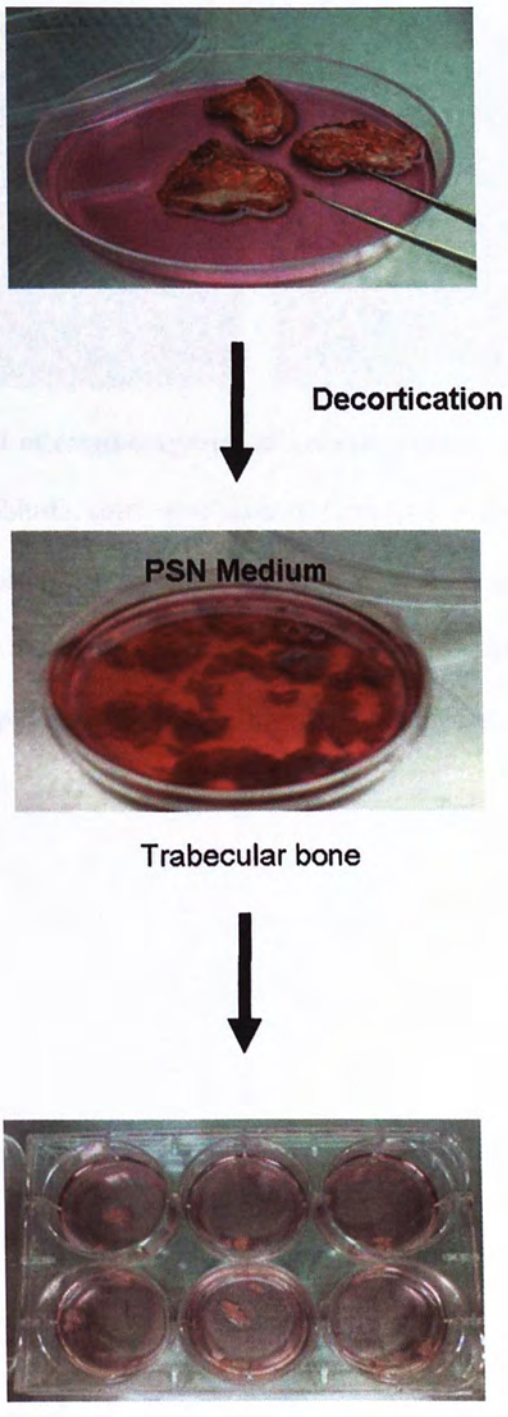

Figure 4.1. Isolation of human osteoblasts from bone biopsies. Bone pieces from normal and AIS patients were collected and isolated from orthopedic surgeries. Unwanted tissues and blood were removed and the bone pieces were cleaned. Trabecular bone pieces were cultured in alpha-modified Eagle's Medium supplemented with 10% FBS, 1% PNS and D-valine for the migration of osteoblasts.

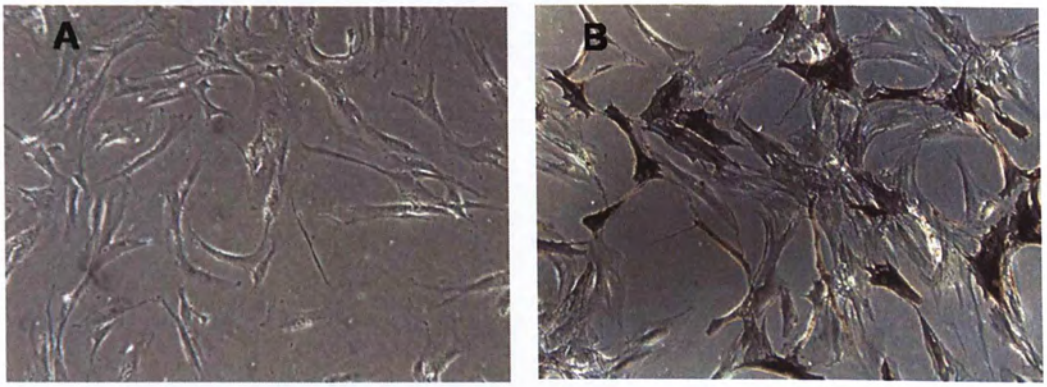

Figure 4.2. Typical microphotograph of normal human osteoblasts isolated in this study. (A) Osteoblastic cells were isolated from bone pieces after culture (x100). (B) After culture in osteogenic medium for 4 days, the cells showed the presence of alkaline phosphatase. ALP activity was assessed by NBT/BCIP staining. Cells that were stained purple indicate a positive pool, as denoted above (100x).

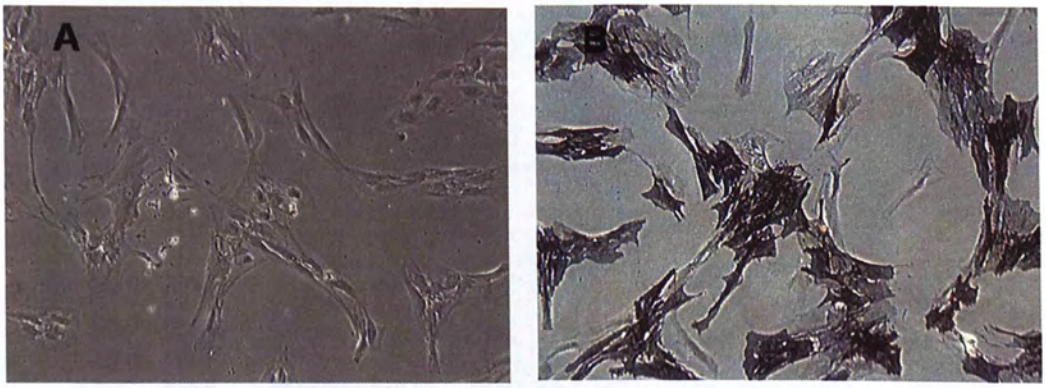

Figure 4.3. Typical microphotograph of human AIS osteoblasts isolated in this study. (A) Osteoblastic cells were isolated from bone pieces after culture (x100). (B) After culture in osteogenic medium for 4 days, the cells showed the presence of alkaline phosphatase. ALP activity was assessed by NBT/BCIP staining. Cells stained purple indicate a positive pool, as denoted above (100x).

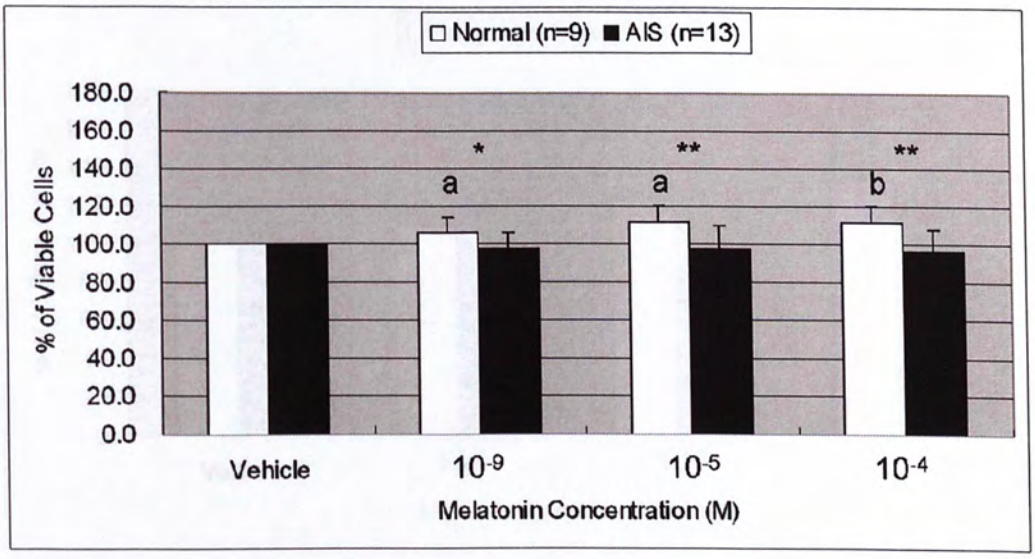

Figure 4.4. Effect of melatonin on the viable cell numbers of normal human and AIS osteoblasts. The effects of melatonin on cell viability were determined by MTT cell viability assay after 3 days of daily melatonin treatments.

a. Student t-test; $p<0.05$ when compared against vehicle

b. Student t-test; $p<0.01$ when compared against vehicle

* Independent Student t-test; $p<0.05$ when Control vs AIS

** Independent Student t-test; $p<0.01$ when Control vs AIS

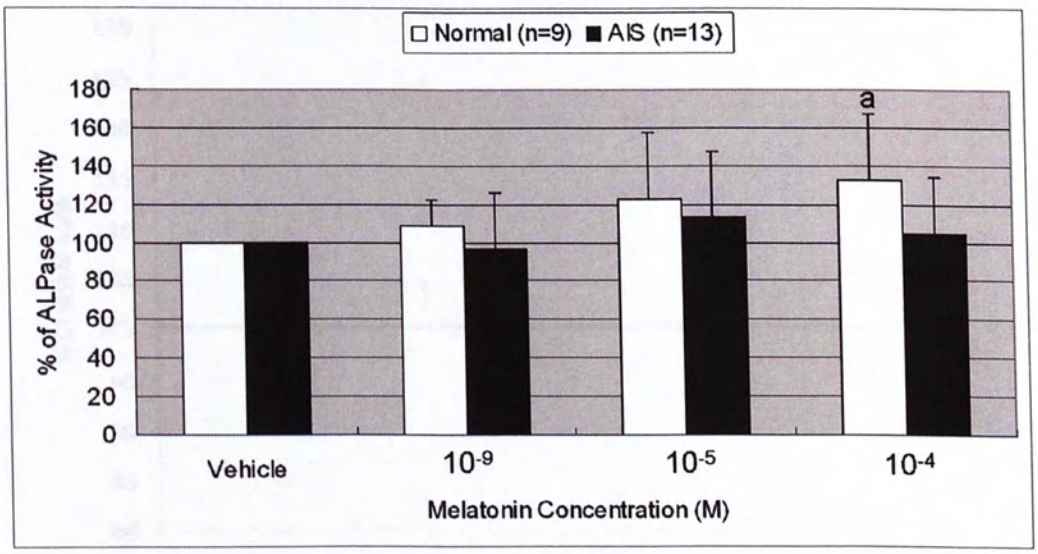

Figure 4.5. Effect of melatonin on the ALPase activity of normal human and AIS osteoblasts. The effects of melatonin on ALPase activity were determined after 3 days of daily melatonin treatments, with normalization by MTT viability assay.

a. Student t-test; $p<0.05$ when compared against vehicle

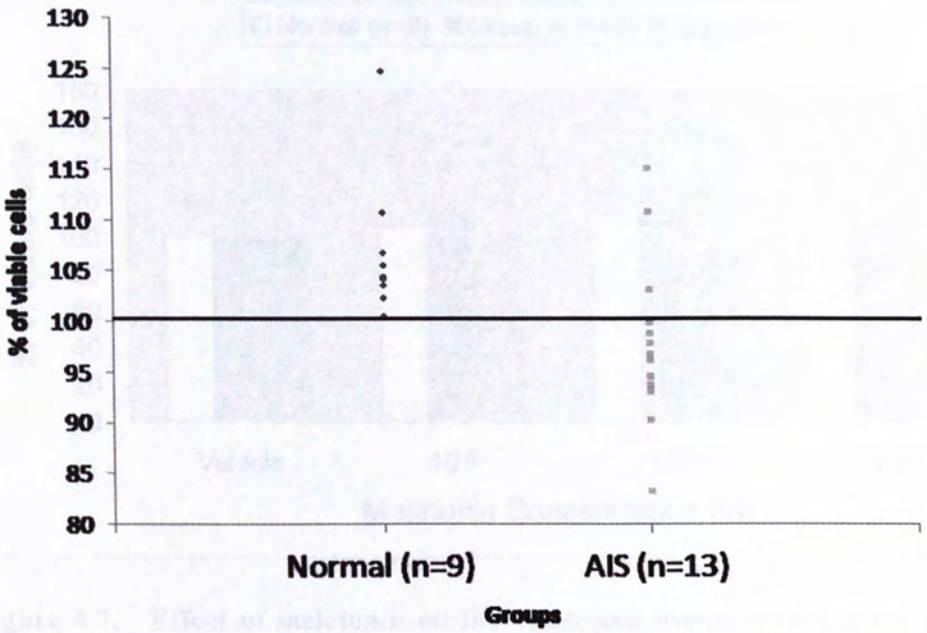

Figure 4.6. Distribution of the viable osteoblast cell numbers in controls and AIS at physiological concentration of melatonin (10^{-9}M). The normal human osteoblasts showed enhancement on cell viability when physicological concentration of melatonin was added. While the AIS osteoblasts, three of the samples showed enhancement on cell numbers when melatonin was added. However, the remaining 10 AIS osteoblasts showed inhibition at physiological concentrations of melatonin.

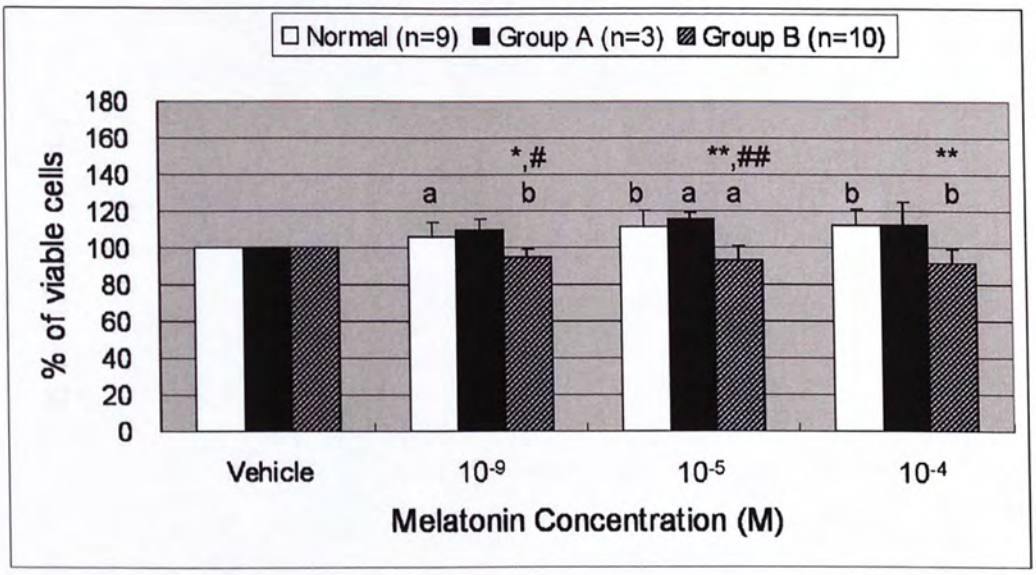

Figure 4.7. Effect of melatonin on the viable cell numbers of normal human and the two groups of AIS osteoblasts. The effect of melatonin on cell viability was determined by MTT cell viability assay after 3 days of daily melatonin treatments.

a. Student t-test; $p<0.05$ when compared against vehicle

b. Student t-test; $p<0.01$ when compared against vehicle

* Post Hoc Test (LSD); $p<0.05$ when Control vs Group B

** Post Hoc Test (LSD); $p<0.01$ when Control vs Group B

\# Post Hoc Test (LSD); $p<0.05$ when Group A vs Group B

\#\# Post Hoc Test (LSD); $p<0.01$ when Group A vs Group B

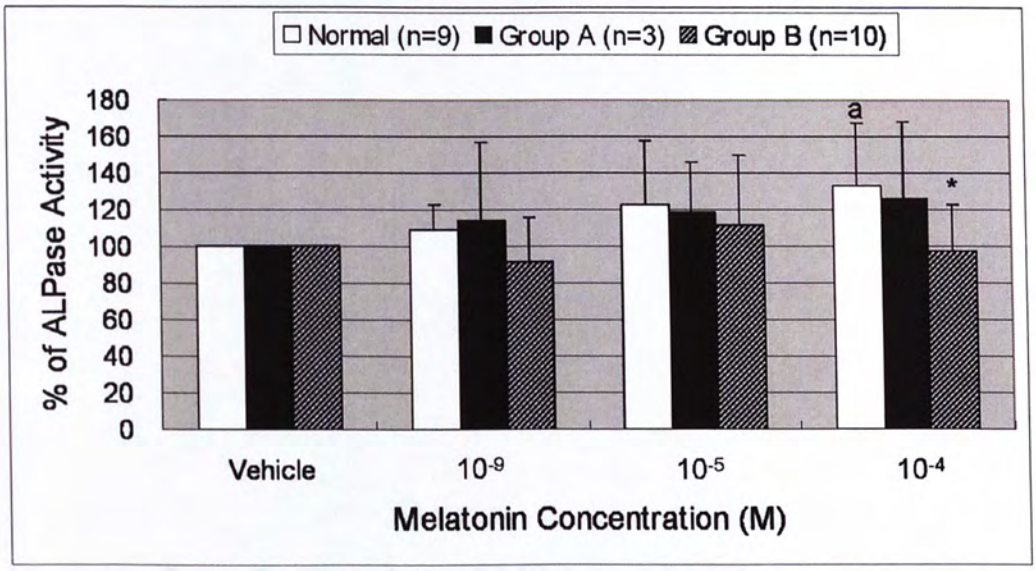

Figure 4.8. Effect of melatonin on ALPase activity in normal human and the two groups of AIS osteoblasts. The effects of melatonin on ALPase activity were determined after 3 days of daily melatonin treatments, with normalization by MTT viability assay.

a. Student t-test; $p<0.05$ when compared against vehicle

* Post Hoc Test (LSD); $p<0.05$ when Control vs Group B

Chapter 5 Expression of MT1 and MT2 receptors in AIS Osteoblasts

5.1. Introduction

Melatonin signaling pathway dysfunctions have been reported in osteoblasts derived from selected group of severe AIS patients (Azeddine *et al.*, 2007, Moreau *et al.*, 2004). In Chapter 4, it has been also demonstrated the presence of abnormal response of osteoblasts to melatonin effect on proliferation and differentiation in subgroups of the AIS. There were two distinct responses from the AIS osteoblasts toward melatonin. One was similar to the normal osteoblasts enhancing effects, namely as Group A. Another was Group B where melatonin was shown to have inhibitory effect on both osteoblasts proliferation (cell viability) and differentiation (ALPase activity). To account for the observed melatonin pathway dysfunction, it is speculated that the abnormality could be related to a quantitative or qualitative variation in the melatonin receptors. Reports have indicated that melatonin receptors could be regulated homologously or heterologously, or by other stimuli such as light or estradiol (Witt-Enderby *et al.*, 2003). It was also shown that G-protein uncoupling, receptor down-regulation, internalization and phosphorylation might be part of the mechanisms for melatonin receptor regulation (Witt-Enderby *et al.*, 2003). In the present study, the nature of melatonin receptors in these AIS osteoblasts was evaluated. This might help to provide further understanding and explanation of the observed heterogeneous responses of the AIS osteoblasts to melatonin.

Three types of mammalian melatonin receptors, MT1, MT2 and MT3, have been identified (Witt-Enderby *et al.*, 2003). However, MT3 receptor has only been identified in avians and rodents, but have not been identified in humans (Nosjean *et*

al., 2001, Reppert, 1997). The two human melatonin receptors, MT1 and MT2, are membrane-associated G-protein-coupled receptor families, and belonging to the rhodopsin superfamily (Morgan *et al.*, 1989, Reppert, 1997, Reppert *et al.*, 1995, Reppert *et al.*, 1994, Rivara *et al.*, 2005, von Gall *et al.*, 2002). Both MT1 and MT2 have been suggested to play a role in bone remodeling (Dubocovich *et al.*, 2001, Reppert *et al.*, 1995, Reppert *et al.*, 1994). Although active expressions of these 2 receptors can also be found in the retina (Fujieda *et al.*, 1999, Meyer *et al.*, 2002, Savaskan *et al.*, 2002) and brain (Kong *et al.*, 2008, Uz *et al.*, 2005), it is also distributed across many other tissues (Achong *et al.*, 2003, Dillon *et al.*, 2002, Lanoix *et al.*, 2006, Naji *et al.*, 2004). In general, the signaling pathway mechanism after the activation of either MT1 or MT2 receptors in osteoblasts could result in the inhibition of adenylate cyclase activity (von Gall *et al.*, 2002), which in turn inhibit forskolin-induced cyclic AMP formation, with a subsequent decrease in activated protein kinase A (Vanecek, 1998). This signal transduction mechanism is the best recognized pathway. However there are speculations on other possible transductions involved in different tissues. For example, in HEK293 cells, melatonin acting through MT1 receptors downregulates the activity of adenylate cyclase, though MT1 receptor can also activate upregulate phospholipase C-β in HEK293 cells (Brydon *et al.*, 1999). Through the MT2 receptor of the HEK293 cells, melatonin can inhibit soluble guanylate cyclase as well (Petit *et al.*, 1999). Depending on the type of cell, melatonin may regulate different secondary messengers within the same signal transduction pathway (New *et al.*, 2003).

In addition to the receptor diversity, Moreau *et al.* (2004) found dysfunction of melatonin signaling pathway in osteoblasts derived from AIS patients. The addition

of melatonin to osteoblasts isolated from a selected group of AIS patients failed to inhibit the forskolin-stimulated cyclic AMP. The degree of inhibitions can be further classified into 3 distinct groups based on the functional response (Moreau *et al.*, 2004). In regards to the possible dysfunction of melatonin signaling pathway, the Gi protein coupling was thought to be dysfunctional in some AIS patients (Letellier *et al.*, 2008, Moreau *et al.*, 2004). In another study at our center, we found that melatonin 1B polymorphism was associated with the occurrence of AIS (Qiu *et al.*, 2007a). In this study, the AIS patients demonstrated a majority with the phenotype of TC (42.8%) and CC (50.6%), with a higher C allele expression (72%) than T allele (28%). However, the MT2 gene was not associated with curve severity. It was suggested that MT2 might be a disease predisposition gene.

Therefore, with the assumption that AIS patients have systemic problem with the melatonin signaling pathway, the present study aims to investigate the expression of the melatonin receptors, MT1 and MT2, in human AIS osteoblasts which to the best of our knowledge has never been properly studied.

5.2. Methodology

5.2.1. Osteoblast Samples

The samples of normal control and AIS osteoblasts were the same as described in the previous chapter (Chapter 4). Methods of collection and isolation are explained in details in Chapter 4.2.

5.2.2. Protein Expression of Melatonin Receptors in AIS Osteoblasts

To investigate the presence of the melatonin receptors in AIS osteoblasts, the protein expression of melatonin receptors were evaluated semi-quantitatively by Western blotting and compared with normal controls.

5.2.2.1. Materials and Reagents

Radioimmunoprecipitation assay (RIPA) Buffer

The following including 1.8 g NaCl (Merck & Co., Darmstadt, Germany), 1.0 g deoxycholic acid (Sigma, St.Louis, USA), 2.0 ml NP-40 (Fluka, Steinheim, Switzerland), 1 ml 20% SDS (Amresco, Solon, USA), and 6.7 ml 1.5M Tris-HCl (pH 8.0) (Sigma, St.Louis, USA) was dissolved in 200 ml distilled water.

2x Sodium Dodecyl Sulfate (SDS) Gel Loading Buffer

Bromophenol blue (4 mg) (Merck & Co., Darmstadt, Germany) was dissolved in 1.6 ml 1M Tris-HCl (pH 6.8) (Sigma, St.Louis, USA), 4 ml 10% SDS (Amresco, Solon, USA), 2 ml glycerol (Amresco, Solon, USA), 1 ml β-mercaptoethanol (Merck & Co., Darmstadt, Germany) and 1.4 ml deionized water. The solution was mixed gently and the final volume made up to 10 ml.

4% Stacking Gel

0.49 ml of 30% acrylamide solution (containing 0.3 g/ml acrylamide and 8 g/ml of N, N'-methylene bisacrylamide) (GE Healthcare, Bucks, UK), 0.97 ml 0.1M Tris-HCl (pH 6.8) (Sigma, St.Louis, USA), 35 µl 10% SDS (Amresco, Solon, USA) and 1.96

ml of deionized water were mixed gently. Prior to loading the gel onto the cask, 4 µl TEMED (Merck & Co., Darmstadt, Germany) and 14 µl 10% ammonium persulfate (Sigma, St.Louis, USA) were added to the mixture.

12% Separating Gel

3.5 ml of 30% acrylamide solution (containing 0.3 g/ml acrylamide and 8 g/ml of N, N'-methylene bisacrylamide) (GE Healthcare, Bucks, UK), 3.3 ml 1M Tris-HCl (pH 8.8) (Sigma, St.Louis, USA), 87.5 µl 10% SDS (Amresco, Solon, USA) and 1.9 ml of deionized water were mixed gently. Prior to loading the gel onto the cask, 4 µl tetramethylethylenediamine (TEMED) (Merck & Co., Darmstadt, Germany) and 35 µl 10% ammonium persulfate (APS) (Sigma, St.Louis, USA) were added to the mixture.

SDS Running Buffer

The following including 3.0 g 0.25M Tris Base (Sigma, St.Louis, USA), 14.4 g 1.92M glycine (Amresco, Solon, USA) and 1.0 g 1% SDS (Amresco, Solon, USA) were dissolved into 800 ml deionized water. The solution was mixed gently and the pH was adjusted to 8.3. The final volume was made up to 1000 ml with deionized water.

Tris-Buffered Saline with Tween-20 (TBST)

The following including 8.8 g NaCl (Merck & Co., Darmstadt, Germany), 0.2 g KCl (Merck & Co., Darmstadt, Germany) and 3 g Tris base (Amresco, Solon, USA) were dissolved in 800 ml distilled water. The solution was mixed gently and 0.5 ml Tween-20 (Amresco, Solon, USA) was added. The pH was adjusted to 7.4 and the

final volume made up to 1000 ml with distilled water. The solution was sterilized and stored at 4°C.

5% Skimmed Milk

Skimmed milk (5.0 g) was dissolved in 100ml TBST. The diluted skimmed milk was mixed gently and stored at 4°C. All the primary and secondary antibodies were diluted with 5% skimmed milk at different dilution factor shown below:

Primary Antibody

Dilution range was determined by serial concentrations of the antibody:

Goat IGg anti-human MT1 receptor (Santa Cruz, Santa Cruz, USA) (Dilution: 1:500)

Goat IGg anti-human MT2 receptor (Santa Cruz, Santa Cruz, USA) (Dilution: 1:200)

Goat IGg anti-human Actin (Santa Cruz, Santa Cruz, USA) (Dilution: 1:500)

Secondary Antibody

Dilution range was determined by serial concentrations of the antibody:

Donkey anti-goat IgG (Santa Cruz, Santa Cruz, USA) (Dilution: 1:500)

Stripping Buffer

Tris-HCl (12.5 g) (pH 6.8, 0.5M) (Sigma, St.Louis, USA) in 20 ml 10% SDS (Amresco, Solon, USA) and 67.5 ml distilled water were mixed gently and, in a fumehood, 0.8 ml ß-mercaptoethanol (Merck & Co., Darmstadt, Germany) was added into the solution.

5.2.2.2. Western Blotting

For protein extraction, the cells were seeded at a cell density of $1x10^6$ cells in a 150 cm^2 flask (Corning, Corning, USA) and cultured in α-MEM containing 10% FBS. Upon reaching confluence, the cells were cultured for 3 more days. Then, the cells were trypsinized and lysed in RIPA buffer containing 1 mM protease inhibitor cocktail (Sigma, St.Louis, USA) for 30 minutes. The mixture was centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was collected for protein detection.

For Western blotting, approximately 16 µl of the prepared supernatant was taken out and mixed with an equal volume of 2x SDS gel loading buffer. The samples were placed in a boiling water bath for 5 minutes. After sonication, the samples were centrifuged at 14,000rpm for 7 minutes. The supernatant (25 µg), in 15ul, was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (BioRad, Hercules, USA) using 12% separating gel and 4% stacking gel for 45 minutes at 200V. The gel was removed from the tank and electroblotted onto a methanol-activated nitrocellulose membrane (Hybond-ECL; GE Healthcare, Bucks, UK) by using a semi-dry method for 30 minutes at 400 mA. After semi-dry transference, the membrane was washed three times with TBST buffer and blocked with 5% skimmed milk for 1 hour at 4°C. Thereafter, it was probed with the selected goat anti-human primary antibodies overnight at 4°C. The membrane was washed three times with TBST buffer and incubated with horseradish peroxidase-conjugated donkey anti-goat secondary antibody for 1 hour at room temperature. After three washes, the immunocomplex was visualized using ECL

Western Blotting Detection Reagents (GE Healthcare, Bucks, UK) and ECL mini-Camera (AlphaImager 2200 Imaging System).

After analyzing the first primary antibody, the same membrane was rinsed with TBST buffer and the antibodies were stripped away by stripping buffer for 30 minutes. Then, it was incubated with anti-actin antibody for 1 hour at room temperature as reference protein. As before, after conjugating with a secondary antibody, it was visualized using ECL Western Blotting Detection Reagents (GE Healthcare, Bucks, UK) and ECL mini-Camera (AlphaImager 2200 Imaging System). An affinity-isolated antigen-specific antibody directed against the actin protein (Santa Cruz, Santa Cruz, USA) was used as a reference protein for semi-quantification of the Western blots. Semi-quantification of the bands was done by an image analytical program, ImageJ (Version 1.37, USA). All results were confirmed in duplications.

5.2.3. Genotyping of MT2 receptors by Restriction Fragment Length Polymorphism (RFLP)

The genotyping of MT2 receptors by RFLP was performed as described previously by Qiu *et al.* (2007a). This was to determine whether there is any difference in the MT2 receptor polymorphism in the AIS patients from this study. In brief, total peripheral DNA was isolated from the 3 ml blood samples of AIS patients using DNA extraction kit (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The forward and reverse primer consisted of the following sequence:

5'-CCGTTCATTGTGTTTCCT-3'

5'-AGACAGTCCTTGGTTTTTTC-3'

Genomic deoxyribonucleic acid (DNA) amplification was performed using a polymerase chain reaction (PCR) with 12.5 μl reaction mixtures, containing 0.5 Units of Taq polymerase, 10 mmol/L of each primer and 2 mmol/L $MgCl_2$. A typical 35 amplification cycles, consisted of 30 seconds, at 96°C, 45 seconds at annealing temperature and 30 seconds at 72°C, was used. The final elongation step consisted of 7 minutes at 72°C. For the restriction enzyme digestion, PCR product was subjected to 3 to 5 Units of the required enzyme, in the presence of the accompanying buffer, was incubated overnight. The restricted PCR products were visualized by electrophoresis in 4% agarose gel. Validation was performed by duplicating the samples.

5.2.4. Clinical Evaluations of the AIS Patients

The anthropometric and BMD measurments of the AIS patients were the same as described in the previous chapter (Chapter 3). Comparison was then made with matched controls data (z-score) from the normal population. Detailed procedures on the measurments are explained in details in Chapter 3.2.

5.2.5. Data Analysis

All data were expressed as mean ± S.D. Statistical significance of the difference between the band intensity of the receptors from Western blotting was analyzed between the control and the three groups by ANOVA. The difference was

considered significant when $p < 0.05$. SPSS software (version 11; SPSS, Inc., Chicago, IL) was used for statistical analysis.

5.3. Results

5.3.1. Semi-quantification of Melatonin Receptors in AIS Osteoblasts

In Figure 5.1 and 5.2, MT1 receptors were found in osteoblasts isolated from AIS patients and normal controls. As for MT2 receptor expressions, it was expressed in all the normal controls (Figure 5.1). However, only 69% of AIS subjects showed expressions of MT2 receptor from their osteoblasts (Figure 5.2). With further analysis of the Western blotting according to the subgroups suggested in Chapter 4, all the subjects in Group A showed observable band of MT2 expression. However, in Group B, only 60% of the patients showed the expression of MT2 receptor on their osteoblasts. Therefore, the Group B subjects could be divided into 2 groups, according to the presence of MT2 expression on osteoblasts as Group B1 and B2, namely.

In Figure 5.3, it showed that there was no significant difference on the MT1 receptor expressions between the normal osteoblasts and with Group A (p=0.236), Group B1 (p=0.977) and Group B2 (p=0.068). There was no significant difference between the MT2 receptor expression of normal controls with Group A (p=0.202) and Group B1 (p=0.138). In comparing the receptor expression intensity between different AIS subgroups, there was no difference between the MT1 expressions in Group A with Group B1 (p=0.406) and Group B2 (p=0.352). The MT2 expression between Group A and B1 was similar (p=0.996).

5.3.2. RFLP

Figure 5.4 shows the gel image of all the genotype results of MT2 receptor of the thirteen AIS subjects. The distribution is summarized in Table 5.1. In Group A (n=3), 67% of the subjects (2 out of 3 subjects) showed the genotype expression of CT, while the only 33% of the subjects showed TT (1 out of 3 subjects). In Group B (n=10), 60% of them demonstrated genotype expression of CT (6 out of 10 subjects). The remaining showed 30% of the subjects (3 out of 10 subjects) carrying the genotype of TT, while 10% was CC (1 out of 10 subjects).

5.3.3. Functional Response Between the Different AIS Groups

Figure 5.5 shows the effect on proliferation between the three different groups of AIS osteoblasts toward melatonin. The osteoblasts from the AIS patients were divided into three groups based on the classification on Chapter 4 and the presence of MT2 receptors. In the first group (Group A; Figure 5.5), melatonin treatment enhanced the number of osteoblasts, similar to the normal control, at all concentrations (10^{-9}, 10^{-5} and 10^{-4} M). The promotion was significant at 10^{-5} M ($p=0.021$). However, the enhancement of osteoblast proliferation at melatonin concentrations 10^{-9} M ($p=0.109$) and 10^{-4} M ($p=0.263$) was not statistically significant. The non-significant association between these two doses of melatonin might be accounted by the large standard deviations in the small sample size (n=3). Both groups in Group B (Group B1 and Group B2, Figure 5.5) showed an inhibition on the number of osteoblasts to effect of melatonin. The inhibition of Group B1 was statistically significant at all concentrations of 10^{-9} ($p=0.039$) and 10^{-4} M

(p=0.039). For Group B2, the inhibition was not significant at concentrations of melatonin (p>0.05). In comparing between the normal controls and Group B1 and B2, there were significant differences in the number of osteoblasts at all melatonin concentrations (p<0.01). There was no significant difference between the number of viable osteoblasts in Group A and normal controls at melatonin concentrations of 10^{-9} (p=0.472), 10^{-5} (p=0.305) and 10^{-4} M (p=0.995). In comparing Group A and Group B1, there were significant differences in the number of osteoblasts at melatonin concentrations of 10^{-9} (p=0.018) and 10^{-5} (p=0.001). However, there was no significant difference at the melatonin concentrations of 10^{-4} (p=0.084). While similarly in the comparison between Group A and Group B2, there was significant difference on the effect of melatonin toward the number of osteoblasts at all concentrations of 10^{-9} (p=0.045), 10^{-5} (p=0.002) and 10^{-4} (p=0.05). Comparing between the two groups in Group B, there was no significant difference on any of the melatonin concentrations (p>0.05).

Figure 5.6 shows the effect of melatonin on ALPase activity between the three different groups of AIS osteoblasts. With the same classification mentioned above, the AIS osteoblasts displayed 3 types of response to various concentrations of melatonin (Figure 5.6). In Group A (Figure 5.6), melatonin treatment promoted ALPase activity, similar to the normal control, at all concentrations (10^{-9}, 10^{-5} and 10^{-4} M). However, the enhancement of ALPase activity in Group A was not statistically significant at any of the melatonin concentrations (p>0.05). For the Group B1 (Figure 5.6), it showed an inhibition on the ALPase activity at all concentrations of melatonin, while for Group B2, it showed an enhancement of ALPase activity. However, there was similar ALPase activity in Group B1 and

Group B2 ($p>0.05$). In comparing between the normal controls and Group B1, there were significant differences in ALPase activity at the melatonin concentrations of 10^{-4}M ($p=0.017$), but not at the melatonin concentrations 10^{-9} M ($p=0.136$) and 10^{-5} M ($p=0.121$). In the comparison between the normal controls and Group B2, no significant difference was shown between the two groups in ALPase activity ($p>0.05$). Similarly, there was no significant difference between the ALPase activity in Group A and normal controls ($p>0.05$). In comparing Group A and the two groups in Group B (Group B1 and Group B2), there was no significant difference in the melatonin effect on ALPase activity ($p>0.05$). There was no significant differences of ALPase activity in response to melatonin between the two subgroups in Group B ($p>0.05$).

5.3.4. Correlation of the Clinical Phenotypes with the Different AIS Subgroups

Table 5.2 shows the results of body weight, height, corrected height, BMI, arm span and BMD of the three AIS groups obtained from the Chapter 3. The AIS patients in Group B2 showed a longer arm span, body weight, height and BMI than those of Group A and Group B1. Also, the dominant and non-dominant sides of the bilateral femoral neck BMD of Group B2 were higher than Group A and Group B1. When adjusted with the age-matched normal community, Group B2 showed a longer arm span, body weight and BMI when compared with Group A and Group B1. Similarly on BMD, the dominant and non-dominant sides of the bilateral femoral neck of Group B2 were higher than Group A and Group B1. The midshaft apparent BMD was lower in the AIS patients of Group B than those in Group A. While, the

other parameters, they all seemed to be similar between the three groups. However, none of the comparisons was significant. The lack of significance might be due to the small sample size. In Table 4.4, the prevalence of having a family history with scoliosis is 75% in Group B2 (3 out of 4 subjects), while Group B1 and Group A have a prevalence of 17% (1 out of 6 subjects) and 0% (0 out of 3 subjects), respectively.

5.4. Discussion

In the present study, it was shown that MT1 receptor protein expression was observed in all the AIS subjects. According to the grouping in Chapter 4, it was shown that all Group A subjects have MT2 expression, while only 60% of the subjects in Group B showed MT2 expression. The genotypes of Group A subject were TC (2/3) and CC (1/3). For the Group B subjects with MT2 expression, the genotypes were TC (4/6), CC (1/6) and TT (1/6). The subjects without MT2 receptor protein expression allowed further subgrouping into Group B1 and B2, namely. The Group B2 patients without MT2 receptor response were found to have a longer arm span than the other two groups of AIS subjects. In addition, the Group B2 patients have higher prevalence rate of positive family history of AIS. Although the result did not reveal direct relationship with the BMD level, the finding might reflect possible association of melatonin receptor abnormality with disproportional endochondral ossification and abnormal bone growth and longer arm span in AIS patients.

As MT2 polymorphism was associated with the occurrence of AIS (Qiu *et al.*, 2007a), the result (Table 5.1 and Figure 5.4) showed 61.5% (8 out of 13 subjects) of

the AIS patients having the majority of the genotype TC. Only 7.7% (1 out of 13 subjects) expressed a rare TT genotype in Group B1, while 30.8% (4 out of 13 subjects) showed CC genotype expression. The frequency of C allele of this single nucleotide polymorphism (SNP) located in the promoter was 61.5%, while the T allele was 38.5%. This result matched with the reported findings of the AIS patients having a majority at the C allele and the genotype of CC and TC (Qiu *et al.*, 2007a). However, the small sample size has to be taken into account. As from the report from Qiu *et al.* (2007a), the majority of the AIS patients carrying genotype CC (50.6%) than genotype TC (42.8%). In that study, the majority of the C allele was 72% in the AIS subjects, while the T allele was 28%.

From the previous chapter, Group B of AIS osteoblasts demonstrated a generalized inhibitory response in proliferation and differentiation to melatonin. However, in this study, not all the Group B showed similar MT2 expression. Even though Group B2 has no MT2 phenotype by Western blotting, melatonin induced inhibitory response in proliferation and differentiation was still found. By genotyping, MT2 receptor gene was also found in Group B2 AIS osteoblasts. Hence, it is speculated that there might be problem with either the gene translation or the translocation of the MT2 receptor to the membrane in the Group B2 osteoblasts. The abnormal MT2 receptor is likely to result in impaired response toward melatonin.

With the new classification, the proliferative response of osteoblasts to melatonin was further divided into three groups of AIS osteoblasts. In Figure 5.5 and 5.6, the proliferative and differentiation response of the three groups of AIS osteoblasts toward melatonin was summarized. Group A showed an enhancement in cell

numbers and ALPase activity with serial concentrations of melatonin, similar to the pattern of normal controls. While for Group B1, it showed inhibitory response in both proliferation and differentiation to melatonin. As for Group B2, it showed inhibition in proliferation to melatonin though a lesser degree than Group B1. In contast to Group B1, minimal enhancement on the ALPase activity was found instead of inhibition. This observation is similar to the finding of Moreau *et al.* (Azeddine *et al.*, 2007, , 2004), who reported 3 distinct groups of AIS osteoblasts based on their responsiveness to melatonin in the cyclic AMP assay. As it is shown in the study of Satomura *et al.* (2007), the inhibition of cyclic AMP would enhance osteoblasts to proliferate under the same melatonin concentrations. Similar findings on lower cyclic AMP accumulation resulting in higher cell proliferation in other cell types (Mallat *et al.*, 1998, Tsuboi *et al.*, 1996, Watson, 1976) have been reported. Hence, the abnormal functional response to melatonin might imply a possible dysfunction in the melatonin signaling pathway. However, it may still be too early to directly compare our results in the proliferation assay with the one used by Moreau *et al.* (cyclic AMP assay). As in our cellular model, melatonin can cross the cytoplasmic membrane in the viable cell to act in the cytosol with MT3 receptor and translocate in the nucleus through interactions with orphan nuclear receptor. While in Moreau's study, the effect from the nucleus is neglected for the cell is lysed for determination on the cyclic AMP accumulation. Moreover, in the current study assessment of cyclic AMP was not included. Further study would be needed to compare and correlate with the response shown in Moreau *et al.*'s study (Azeddine *et al.*, 2007, , 2004).

With this assumption, multiple studies have identified different loci of susceptibility

(Chan *et al.*, 2002, Salehi *et al.*, 2002, Wise *et al.*, 2000). Similar to Moreau *et al.* finding (Azeddine *et al.*, 2007, , 2004), the different responses in osteoblasts from AIS patients to melatonin would most likely to be a cell autonomous defect rather than a consequential response from the spinal deformity. The author, Moreau *at al.*, commented that abnormality in melatonin signaling pathway of this selected group of AIS could be due to the G protein coupling disturbance (Letellier *et al.*, 2008, Moreau *et al.*, 2004). However, the result of Western blot in our study opened up another explanation that the loss of MT2 receptor function might also play an important role in explaining the abnormality observed in Group B2.

To investigate the effect of MT2 on the signaling process in the cell, luzindole is a commonly used MT2 receptor antagonist (Dubocovich *et al.*, 1998, Satomura *et al.*, 2007, Soares *et al.*, 2003). In a study conducted by Sanchez-Hidalago *et al.* (2007), they investigated whether melatonin can inhibit fatty acid-induced triglyceride (TG) accumulation in ROS17/2.8 cells, a rat osteosarcoma cell line. In a part of their study, luzindole was added in an attempt to elucidate the mechanism of action by which melatonin can influence TG accumulation. The result showed a significant increase in TG accumulation. The increase in TG accumulation, which antagonize the osteoblastic pathway (Ahdjoudj *et al.*, 2001, Diascro *et al.*, 1998), was shown to inhibit the stimulatory effect of melatonin on the osteosarcoma cell line to proliferate and differentiate. Radio *et al.* (2006), showed that melatonin, acting through MT2 melatonin receptors could enhance ALPase activity to osteogenic media alone in mesenchymal stem cells. With MT2 antagonists, 4-phenyl-2-propionamidotetralin (4-P-PDOT) and luzindole, the ALPase activity of the stem cells in osteogenic medium and melatonin was inhibited. These results showed that MT2 receptor

could play an important role in influencing osteoblasts proliferation and differentiation.

The anthropometric data of the 4 Group B2 AIS patients with abnormality of MT2 receptor expression revealed significantly longer arm span, taller in height, heavier body weight and higher BMI than all the other AIS patients in Group A and Group B1. After adjusted for age and compared with normal adolescents (Table 5.3), an even longer arm span and higher BMI difference in Group B2 than Group B1 and Group A AIS could be seen. This could imply that the abnormal expression of MT2 receptors might have a direct effect on the endochondral ossification in the AIS patients. In addition, the Group B2 patients seemed to have a higher prevalence in familial history (Table 5.4). The rate of having first degree family members with scoliosis in Group B2 was 75%, which was much higher than the other two groups. It is likely that AIS patients with long arm span might have a higher prevalence of positive family history.

Group B2 was found to have higher mean body mass index and a higher BMD at both femoral necks than the other two groups of AIS. Also, with age-adjusted comparison with the normal community (Table 5.3), BMD of the dominant and non-dominant femoral neck were higher in Group B2 than Group A and Group B1. Previous studies have demonstrated that body mass and bone mass are directly related, and many studies have shown a positive association of BMD with body mass (Felson *et al.*, 1993, Glauber *et al.*, 1995, Khosla *et al.*, 1996, Lim *et al.*, 2004, Lindsay *et al.*, 1992, Stewart *et al.*, 2002). Although mechanical loading may contribute to this, other factors have been reported to be involved, such as estrogens

(Olson *et al.*, 2008, Yu *et al.*, 2002) and leptin (Cock and Auwerx, 2003). As estrogens are known to play a role toward bone formation (Olson *et al.*, 2008, Yu *et al.*, 2002), the effect of melatonin on bone formation might not be direct. Indirect actions can be induced by secretion of growth hormone (Forsling *et al.*, 1999) and cortisol (Cagnacci *et al.*, 1995, Kostoglou-Athanassiou *et al.*, 1998), or down-regulation of the receptor activator of NF-kappaB ligand (RANKL)-mediated osteoclast formation/activation (Koyama *et al.*, 2002) stimulated by melatonin. Recent studies suggest that estrogens suppress the synthesis of AA-NAT protein (Hayashi and Okatani, 1999, Weiss and Crayton, 1970), the rate-limiting enzyme in the synthetic pathway of melatonin (Illnerova *et al.*, 1983, Klein *et al.*, 1971). Further study on estrogens level of these AIS subjects could be helpful. In *in vitro* study, melatonin may enhance bone formation by suppressing osteoclasts (Koyama *et al.*, 2002, Suzuki and Hattori, 2002) through free radical scavenging properties or enhancing osteoblast activity (Radio *et al.*, 2006, Roth *et al.*, 1999) through specific melatonin receptors (Moreau *et al.*, 2004, Radio *et al.*, 2006). A previous study using goldfish scales had found significant decrease on the tartrate-resistant acid phosphatase, a biomarker of osteoclast activity, after incubating with melatonin (Suzuki and Hattori, 2002). Thus, further signifying possibilities that other melatonin-mediated mechanisms might be involved in bone mineralization (Roth *et al.*, 1999, Satomura *et al.*, 2007).

Although the cellular response in Group A showed similar response to the normal controls, the AIS patients still showed abnormal phenotypic expression of a longer arm span and lower BMD than the normal controls. This may indicate a different etiology involved on the scoliosis occurrence in this group of AIS. This is likely

that the AIS patients consist of a heterogenous population with different etiologies. With this, it may help to explain why osteopenia does not occur in all AIS patients. Most importantly, this study provided another important piece of evidence on the role of melatonin in the etiopathogenesis of AIS.

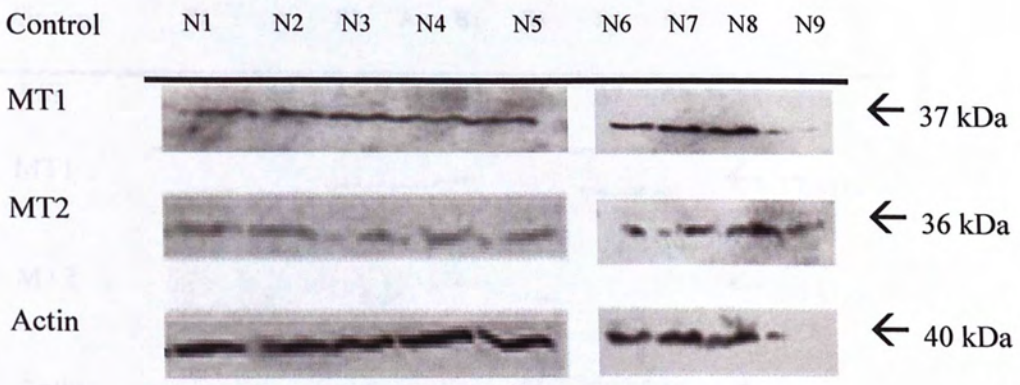

Figure 5.1. Expression of MT receptors in normal human osteoblasts. The cells isolated from 9 normal controls were cultured until they reached confluence. The cells were lysed and examined for the expression of MT1 and MT2 protein receptor expressions by Western blot analysis. All the normal osteoblasts showed the presence of MT1 and MT2 receptor expressions.

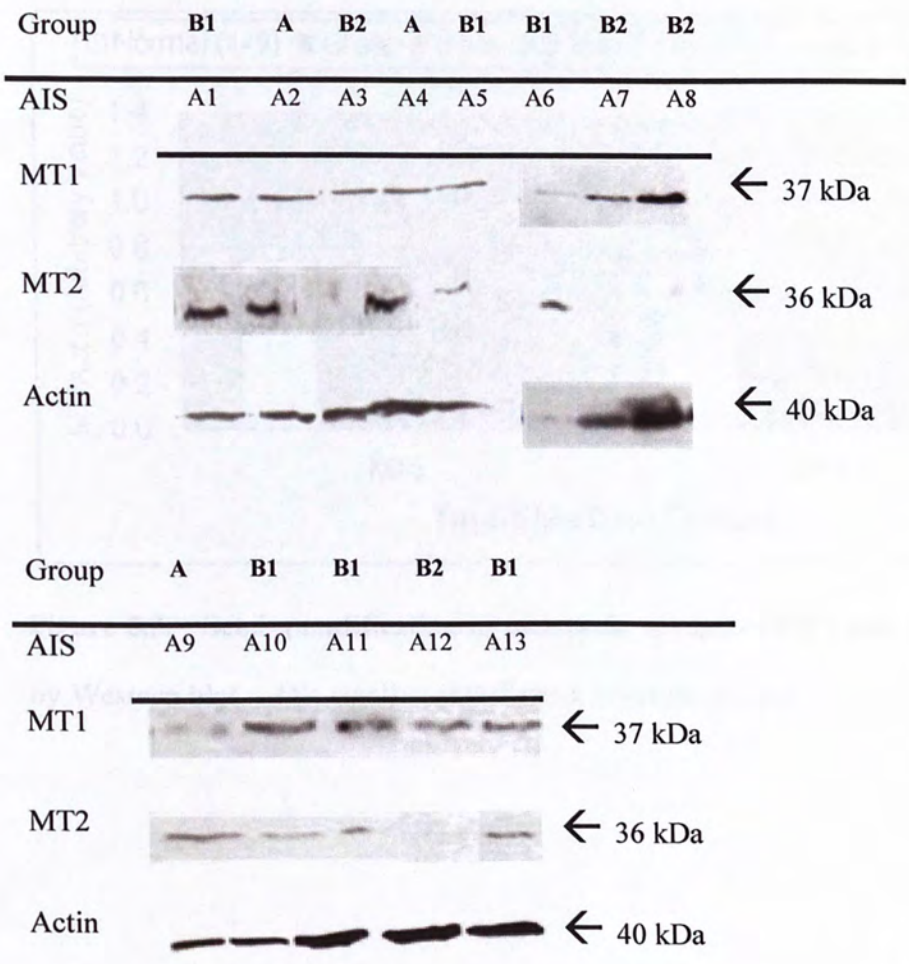

Figure 5.2. Expression of MT receptors in AIS osteoblasts. The cells isolated from 13 AIS patients were cultured until they reached confluence. The cells were lysed and examined for the expression of MT1 and MT2 protein receptor expressions by Western blot analysis. MT1 receptor expression was detected in all AIS osteoblasts. However, in the MT2 receptors, only 9 (Group A and Group B1) out of 13 AIS osteoblasts showed MT2 receptor expression. 4 subjects (Group B2) did not reveal any MT2 receptors expression.

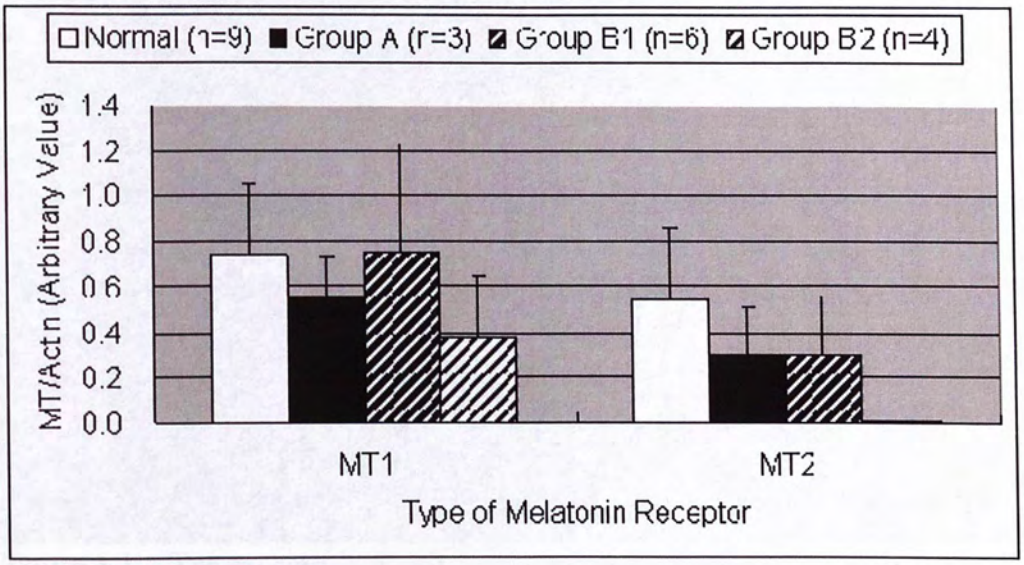

Figure 5.3. Semi-quantification of melatonin receptor (MT1 and MT2) levels by Western blot. (No significant difference between groups).

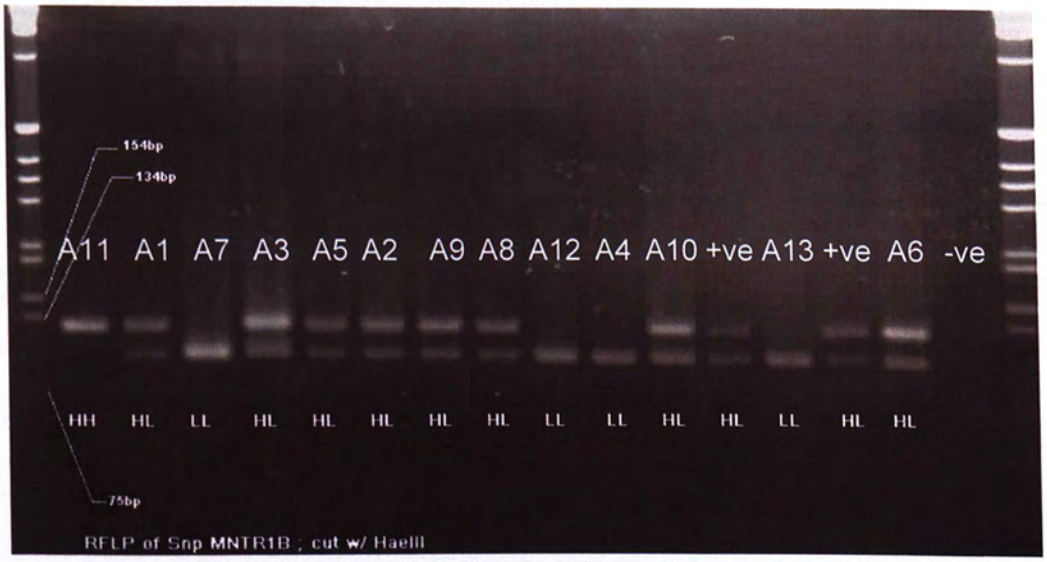

Figure 5.4. The expression of MT2 gene polymorphism in osteoblasts from AIS patients. Peripheral bloods were collected from 13 severe AIS patients. DNA was isolated from the blood and restriction fragment length polymorphism was performed. The tagSNP used was rs4753426, cut with the enzyme HaeIII. The PCR products were separated and visualized on a 4% agarose gel.

Labels: –ve: Negative Control, +ve: Positive Control.

Table 5.1. The Genotype of the SNP rs4753426 in the MT2 Gene of the 13 AIS patients.

	Normal (n=304)*		Group A (n=10)		Group B (n=10)	
Rs4753426	N	%	N	%	N	%
Genotype						
TT	24	7.9%	0	0%	1	10.0%
TC	151	49.7%	2	66.7%	6	60.0%
CC	129	42.4%	1	33.30%	3	30.0%

MT2, melatonin receptor 1B; AIS, adolescent idiopathic scoliosis.
*Normal referenced from Qiu *et al.* 2007 (2007a)

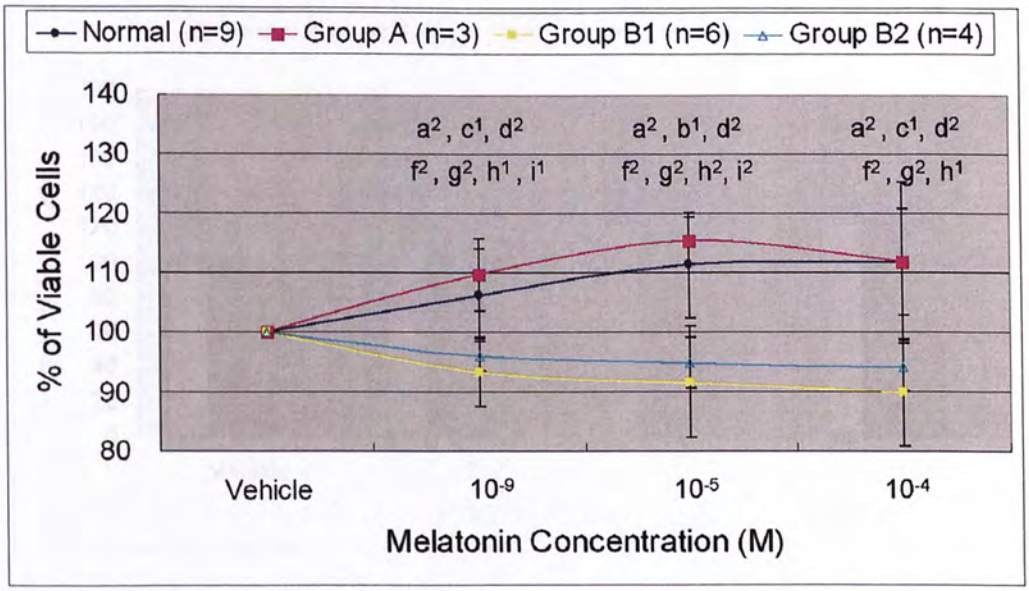

Figure 5.5. Effect of melatonin on the viable cell numbers of normal human and the 3 subgroups of AIS osteoblasts. The effects of melatonin on cell viability were determined after 3 days of daily treatments.

Student t-test; a^1: $p<0.05$, a^2: $p<0.01$, when compared Control against vehicle

Student t-test; b^1: $p<0.05$, b^2: $p<0.01$, when compared Group A against vehicle

Student t-test; c^1: $p<0.05$, c^2: $p<0.01$, when compared Group B1 against vehicle

Student t-test; d^1: $p<0.05$, d^2: $p<0.01$, when compared Group B2 against vehicle

Post Hoc Test (LSD); f^2: $p<0.01$, when Control vs Group B1

Post Hoc Test (LSD); g^2: $p<0.01$, when Control vs Group B2

Post Hoc Test (LSD); h^1: $p<0.05$, h^2: $p<0.01$, when Group A vs Group B1

Post Hoc Test (LSD); i^1: $p<0.05$, i^2: $p<0.01$, when Group A vs Group B1

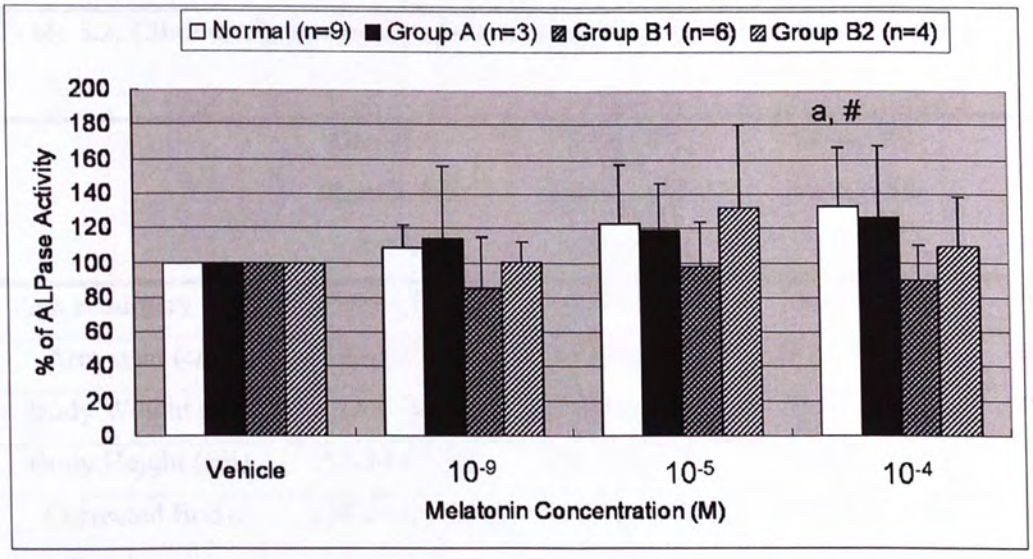

Figure 5.6. Effect of melatonin on ALPase activity in normal human and the 3 subgroups of AIS osteoblasts. The effects of melatonin on ALPase activity were determined after 3 days of daily treatments.

a. Student t-test; $p<0.05$ when compared normal control against vehicle

\# Post Hoc Test (LSD); $p<0.05$ when Control vs Group B1

Table 5.2. Clinical Characteristics between the Three Groups of AIS Patients

	Group A Mean ± SD (n=3)	Group B1 Mean ± SD (n=6)	Group B2 Mean ± SD (n=4)	p-value (a)
Age at surgery (yrs)	15.03 ± 2.30	14.58 ± 1.51	15.55 ± 2.44	0.784
Arm span (cm)	158.83 ± 5.53	159.94 ± 6.29	167.07 ± 5.09	0.153
Body Weight (kg)	45.67 ± 6.36	45.40 ± 4.55	51.95 ± 8.82	0.330
Body Height (cm)	157.20 ± 5.32	156.56 ± 4.89	159.62 ± 4.69	0.649
Corrected Body Height (cm)	158.87 ± 5.36	158.56 ± 4.92	161.40 ± 4.64	0.675
BMI (kg/m^2)	18.03 ± 1.36	17.82 ± 1.97	18.52 ± 2.15	0.860

a: The p value indicated any inter-group difference by One-way ANOVA.

Statistical significance at $p<0.05$

Table 5.3. Z-Score Comparison on the Anthropometry and Bone Geometry Between the Three Groups of AIS Patients

	Group A Mean ± SD (n=3)	Group B1 Mean ± SD (n=6)	Group B2 Mean ± SD (n=4)	p-value (a)
Arm span (cm)	0.25 ± 0.90	0.44 ± 0.93	1.40 ± 0.65*	0.185
Body Weight (kg)	-0.45 ± 1.20	-0.45 ± 0.67	0.51 ± 1.37	0.385
Body Height (cm)	-0.11 ± 1.17	0.91 ± 2.81	0.27 ± 0.89	0.775
Corrected Body Height (cm)	0.45 ± 1.21	1.81 ± 2.98	0.97 ± 0.84	0.672
BMI (kg/m^2)	-0.75 ± 0.78	-0.83 ± 0.90	-0.55 ± 1.03	0.898
Non-dominant Femoral Neck BMD (g/cm^2)	-0.51 ± 1.16	-0.77 ± 0.64	-0.26 ± 1.07	0.716
Dominant Femoral Neck BMD (g/cm^2)	-0.28 ± 0.71	-0.82 ± 0.61*	-0.10 ± 0.73	0.295
Femoral Neck (Convex) BMD (g/cm^2)	-0.16 ± 0.85	-0.77 ± 0.62	-0.38 ± 0.84	0.537
Femoral Neck (Concave) BMD (g/cm^2)	-0.63 ± 1.02	-0.83 ± 0.62*	0.02 ± 0.92	0.342
Midshaft Cortical BMD (g/cm^3)	0.21 ± 0.40	-0.50 ± 0.74	-0.21 ± 1.94	0.746
Midshaft Apparent BMD (g/cm^3)	0.11 ± 0.43	-0.62 ± 1.00	-0.03 ± 1.70	0.655

a: The p value indicated any inter-group difference by One-way ANOVA.

Statistical significance at $p<0.05$

*: One sample t-test: $p<0.05$ when AIS vs Normal Population

**: One sample t-test: $p<0.01$ when AIS vs Normal Population

Table 5.4. Familial Occurrence in the Three Groups of AIS Patients

	Group A (n=3)	Group B1 (n=6)	Group B2 (n=4)
Rate of Occurrence	0 % (0/3)	17 % (1/6)	75 % (3/4)

Chapter 6 Summary and Conclusion

6.1. Summary and Discussion

This series of study confirmed the presence of abnormal responses of AIS osteoblasts to melatonin. The present study aimed to investigate the response of cellular activities of osteoblasts from severe AIS girls under the influence of melatonin in different concentrations. The objectives were 1) to study the anthropometric parameters and the bone mineral status of severe AIS girls when compared with control subjects; 2) to document the response of the cellular proliferation and differentiation of osteoblasts from AIS subjects to melatonin at different dosage 3) to study the expression of the melatonin receptors (MT1 and MT2) in normal human and AIS osteoblasts. It was demonstrated that the severe AIS girls had longer arm span and lower BMD of femoral neck in general. There was no significant difference on the enhancement on proliferation and differentiation to melatonin. By comparing between normal controls and AIS osteoblasts, there were significant differences in all concentrations of melatonin, and marginal for high dosage in differentiation. For the anthropometric measurements and BMD of severe AIS girls and healthy controls, the results showed that the severe AIS girls have a significantly longer arm span and lower body mass index than the normal controls. BMD of the femoral neck in the severe AIS girls were significantly lower than that of the normal controls.

However, with a more detail analysis, it could be grouped into 2 subgroups of AIS patients based on the response to physiological dosage of melatonin on cell proliferation. One group (Group A) was similar to the normal control subjects that both proliferation (Figure 4.6) and differentiation (Figure 4.7) of osteoblasts were

enhanced when melatonin was added. On the contrary, the osteoblasts of the other group (Group B) showed an inhibitory effect of melatonin on cell proliferation (Figure 4.6) and cell differentiation (Figure 4.7). With further investigation on the expression of melatonin receptors in those AIS subjects, it was shown by Western blotting that melatonin receptor MT2 was not expressed in 4 out of 10 subjects in Group B. This helps to further subgroup the Group B into those with (Group B1) and without (Group B2) MT2 expression. With the limited number of subjects studied in the present study, the anthropometric data of the 4 AIS patients in Group B2 showed significant longer arm span, heavier body weight and higher BMI than all the other AIS patients in Group A and Group B1. Although all the AIS demonstrated a lower BMD than the normal community, Group B2 have a higher BMD of bilateral femoral neck than the other two subgroups of AIS.

With the 3 subgroupings, the response of the osteoblasts in proliferation and differentiation to melatonin was re-analyzed (Chapter 5). Group A showed an enhancement in cell proliferation and ALPase activity with different concentrations of melatonin, similar to the pattern of normal controls (Figure 4.4). Group B1 showed inhibitory response in proliferation and differentiation to melatonin. Group B2 showed inhibition in proliferation to melatonin. However, the degree of inhibition by melatonin is less than Group B1. In contrast to Group B1, Group B2 showed minimal enhancement on the ALPase activity with addition of melatonin. This observation was similar to the finding of Moreau *et al.*, who reported 3 distinct groups of AIS osteoblasts based on their responsiveness to melatonin using cyclic AMP assessment. The study of Satomura *et al.* showed that inhibition of cyclic AMP could enhance proliferation of osteoblasts with the same melatonin

concentrations. This finding supported our speculation that the three subgroups might have similar responses in the cyclic AMP accumulation as shown in Moreau *et al.* study. The abnormal functional response to melatonin may in fact be due to the impaired melatonin signaling pathway.

In the phenotypic expression of the subgroups, the anthropometry of the AIS girls (Group A, B1 and B2) showed longer arm span, lower body mass index and lower BMD than the average adolescent girls. The 4 Group B2 AIS patients with low MT2 receptor expression were found to have significant longer arm span, heavier body weight and higher BMI than the other AIS patients in Group A and Group B1. This implies that the MT2 abnormality might have a direct effect on the endochondral ossification in the AIS patients. Also, the BMD at dominant and non-dominant sides of the bilateral femoral neck were smaller in Group B than Group A. This may indicates a possible difference on the bone material property in Group B. In addition, Group B2 patients also had higher prevalence of positive familial history (75%) than Group A (0%) and Group B1 (25%). The major comparison between the three AIS subgroups is summarized in Table 6.1.

In summary, this study provides important evidence on the hetereogenity of the AIS population in response to melatonin. One of the groups (Group A) showed enhancement in proliferation and differentiation to melatonin, while the other showed inhibition (Group B). With the MT2 receptor analysis, it can further subgroup the Group B into those with (Group B1) and without (Group B2) the presence of MT2 receptor. Based on the previous findings on the dysfunction of the melatonin signaling pathway, the three groups of AIS osteoblasts from our study

might demonstrate similar responses with the groups mentioned in those studies. Also, these three subgroups of AIS patients showed heterogeneous difference in the anthropometric measurement and bone mineral status. Although the AIS patients showed a longer arm span and lower BMI than the controls, Group B2 has the longest arm span and lowest BMI among the three groups of AIS patients. This finding correspond to multiple reports of AIS patients of being more slender and leaner than normal healthy controls. Similarly, the AIS patients showed low BMD when compared with the normal populations. However, the AIS patients in Group B2 showed a higher BMD at the dominant and non-dominant side of the bilateral femoral neck than the other two groups. Group B1 had the lowest BMD among the three groups of AIS patients. This may imply that the bone material property in these three groups might be different. The observations might help to explain why only 30% of the AIS patients were clinically reported to have osteopenia. Although the cellular response in Group A showed similar response to the normal controls, the AIS patients still showed abnormal phenotypic expression of a longer arm span and lower BMD than the normal controls. This may indicate that AIS is likely to be a heterogeneous condition with different etiologies and that the occurrence and progression could be affected by different combination of factors intrinsically and extrinsically. Most importantly, the series of studies reported in this thesis has helped to provide better understanding of the role of melatonin in the etiopathogenesis of AIS.

6.2. Limitations and Further Studies

There are a number of limitations in the present studies. From the results, the

disturbance in endochondral ossification of the severe AIS subjects is reflected in longer arm span and lower BMI. However, this study has not analyzed the possible bone markers and hormonal changes affecting endochondral ossification and membranous ossification of the severe AIS subjects.

In the study on BMD, both femoral necks showed significantly lower BMD than normal controls. Although difference in the BMD of the cortical radius of the midshaft was not significant, the numerical value of the AIS patients was lower than the control. The results indicated a possible abnormality in the property of bone material in the AIS patients. The biomechanical property, material property and the microarchitectural properties have not been studied in this series.

In the *vitro* assessment, the age spectrum of the control was older than the AIS patients. As mentioned by few authors, an increase in age could account for the decrease in expression of melatonin receptors although the effect would be unlikely to affect the results in a negative way. As mentioned before, for practical and ethical reasons, the gender of the normal control consisted of a mixture of male and female patients. While for the AIS osteoblasts, they were all isolated from female patients. The fluctuation of the levels of hormones affecting bone metabolism, such as estrogen, is at the highest during the pubertal stage, especially in females. However, as the study was at the cellular level, the level of hormones, besides melatonin, was adjusted by the culture medium to be similar for all the osteoblasts, both male and female, used in this study.

For the assessment of cellular proliferation, MTT assay only detects the number of

viable cells. Hence, a better approach for detecting proliferation would be by thymidine uptake by the cells. However, as this assessment is radioactive, there was a concern involved on using radiation in the current study. Also, assessments on the possible gene expressions of bone markers should be further assessed in future studies. As melatonin is incorporated to two different responses in AIS osteoblasts, there might be changes on the expressions of different bone markers (e.g. Osteopontin, osteocalcin). For the semi-quantification of melatonin receptors, the lack of MT2 response in some of the AIS osteoblasts of group B might be due to structural difference (mutations) of melatonin receptors thus affecting the binding affinity toward melatonin resulting in the abnormal functional responses. Future studies on correlating cyclic AMP assay with MT2 receptors might help to further delineate the actual defect or dysfunction in the melatonin signaling pathway.

Overall, as this study was the beginning of a a small scale pilot investigation on the effect of melatonin on AIS osteoblasts, larger sample series in future could allow more indepth studies to better document the different responses of the osteoblasts to melatonin and their association with the abnormal phenotypes observed in AIS patients.

6.3. Conclusion

Overall, this is the first report on the abnormal cellular responses of AIS osteoblasts to melatonin. In general, there was no significant response on proliferation and differentiation in the AIS osteoblasts to melatonin. For the anthropometric measurements and BMD of severe AIS girls and healthy controls, the results showed

that the severe AIS girls have a significantly longer arm span and lower body mass index than the normal controls. Based on the responses toward melatonin, the AIS osteoblasts were divided into three groups. The groups of AIS osteoblasts (Group 1 and Group 2), with inhibitory functional responses toward melatonin, have shown disturbance on the phenotypic expression in the AIS patients, such as abnormal anthropometry and lower bone mineral status. With regards to the dysfunction of the melatonin signaling pathway, Group B2 without MT2 expression may play a role toward the abnormal bone growth of having longer arm span. From this study, it is likely that AIS patients are, in actual fact, a heterogenous group with different etiologies. Nevertheless, this study is a good step forward in providing additional interesting observations and understanding of the role of melatonin in the etiopathogenesis of AIS. However, further studies would be needed to determine the possible mechanisms involved in the heterogeneous responses to melatonin of AIS osteoblasts, and their association with abnormal bone growth and phenotypes in AIS patients.

Table 6.1. Summary on major characteristics between the 3 groups of AIS osteoblasts in this study.

	Group A (n=3)	Group B1 (n=6)	Group B2 (n=4)
Genetics			
Subjects with CC genotype	1/3	1/6	2/4
% of Family History with Scoliosis	0.0%	16.6%	75.0%
Protein Expression			
Presence of MT1	Yes	Yes	Yes
Presence of MT2	Yes	Yes	No
Functional Characteristics			
Effect of Melatonin on Proliferation	Enhancement, similar to normal controls	Inhibition	No response
Effect of Melatonin on Differentiation	Enhancement, similar to normal	Inhibition	No response
Phenotypic Characteristics			
Arm span #	Shortest	Moderate	Longest*
BMI #	Moderate*	Lowest*	Highest
Bilateral Femoral Neck BMD #	Moderate	Lowest*	Highest

*Significant difference against the normal population
Compared between the 3 subgroups

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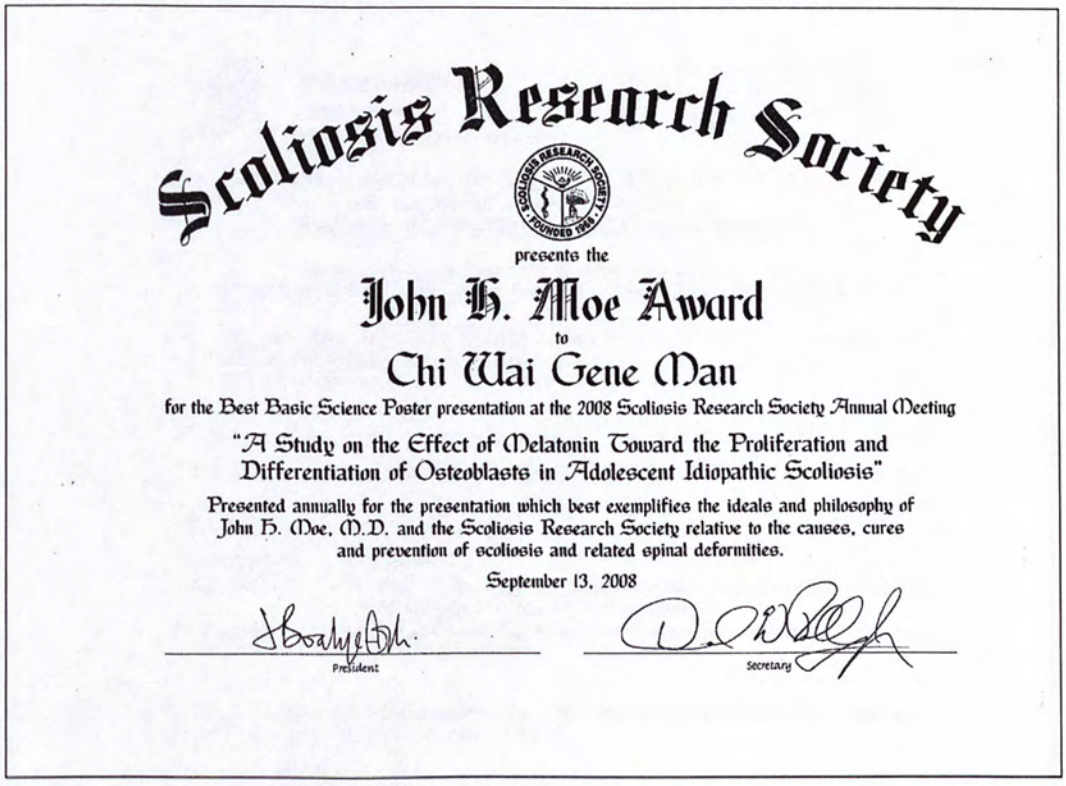
Appendix I



Scoliosis Research Society

presents the

John H. Moe Award

to

Chi Wai Gene Man

for the Best Basic Science Poster presentation at the 2008 Scoliosis Research Society Annual Meeting

"A Study on the Effect of Melatonin Toward the Proliferation and Differentiation of Osteoblasts in Adolescent Idiopathic Scoliosis"

Presented annually for the presentation which best exemplifies the ideals and philosophy of John H. Moe, M.D. and the Scoliosis Research Society relative to the causes, cures and prevention of scoliosis and related spinal deformities.

September 13, 2008

President

Secretary

Appendix II

香港中文大學醫學院
Faculty Of Medicine
The Chinese University Of Hong Kong

醫院管理局
新界東醫院聯網
Hospital Authority
New Territories East Cluster

Joint The Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee
香港中文大學–新界東醫院聯網 臨床研究倫理 聯席委員會

Flat 3C, Block B, Staff Quarters, Prince of Wales Hospital, Shatin, HK
Tel : (852) 2632 3935 Fax : (852) 2646 6653 Website : http://www.crec.cuhk.edu.hk

To: Prof. Jack Chun Yiu CHENG (Principal Investigator)
Dept. of Orthopaedics and Traumatology
Prince of Wales Hospital

3 April 2007

Ethics Approval of Research Protocol

CREC Ref. No.:	**CRE-2007.123**
Date of Approval:	**03 April 2007***
Protocol Title:	**Is Melatonin Signalling Pathway Dysfunction An Important Key Player in the Etiopathogenesis of Adolescent Idiopathic Scoliosis?**
Investigator(s):	**Jack Chun Yiu CHENG, Tzi Bun NG, Yong Qiu, Alain MOREAU, Kwong Man LEE and Hiu Yan YEUNG**

I write to inform you that ethics approval has been given to you to conduct the captioned study in accordance with the following document(s) submitted:

- Research Proposal
- Participant Consent Form in English and Chinese Version
- Participant Information Sheet in English and Chinese Version
- Bone Mineral Density Measurement Consent Form in English Version
- Bone Mineral Density Measurement Consent Form in Chinese Version

This ethics approval* will be valid for 12 months. Application for further renewal can be made by submitting the Ethics Renewal and Research Progress Report Form to the CREC (Download the electronic form template from the http://www.crec.cuhk.edu.hk or http://ntec.home/Research%20Ethics/main.asp). It will be much appreciated if the completion of the project will be reported to the Committee in due course.

The Joint CUHK-NTEC Clinical Research Ethics Committee serves to confirm that research complies with the Declaration of Helsinki, ICH GCP Guidelines, local regulations, HA and University policies.

(Prof. Joseph Lau)
Secretary, Joint CUHK-NTEC
Clinical Research Ethics Committee

c.c. Ms. Alice Ngan – RTAO (Ref. Earmarked Grant 2007/2008)
Encl. CREC/CT0001 – w.e.f. 4/2003 for HA(NTEC) employee concerned ONLY
JL/ei

Appeddix III

香港中文大學矯形外科及創傷學系
Department of Orthopaedics & Traumatology
The Chinese University of Hong Kong

青少年特發性脊柱側彎研究計劃
Adolescent Idiopathic Scoliosis Research Study

參與者須知
Patient Information Sheet

目的:
Objective:

透過身體測量、骨質密度、基因測試、及生物化學測試,對青少年特發性脊柱側彎作尋原的科學研究。

Through bone mineral density measurement, genetic analysis, biochemical analysis, and body measurement to study the etiology of Adolescent Idiopathic Scoliosis

研究計劃內容: 此研究計劃為時2年。
Content of the Study: This study lasts for 2 years.

研究小組將會進行以下檢查:
Research group will carry out the following measurement:

身體測量
Body anthropometric measurement

肢體的骨質密度 (注意: 作骨質密度檢驗,如已懷孕,並在最後一次月經期起計之28天後,接受雙總量X-光骨質密度儀,可能會引致胎兒畸形。)
Bone mineral density measurement of long bones. (Possible risk of radiation induced malformation to the embryo/fetus after 28 days following the onset of the last menstrual period.)

基因測試 (將抽取10毫升血液作測試)
Genetic analysis (10mL venous blood is taken for the analysis)

生物化學測試 (將請參加者提供皮膚及骨樣本作測試)
Biochemical Analysis (Participants are request to provide skin and bone sample for analysis)

對象:
Participants:

1. 年齡十二歲或以上青少年女性
   10 to 16 years of age female
2. 年齡十二歲或以上患有特發性脊柱側彎青少年女性
   10 to 16 years of age female with Adolescent Idiopathic Scoliosis

備註:
Note:

所有資料只作研究用途,絕對保密
All the information collected will be strictly used for research purposes only and will be kept confidential by the researchers.

計劃負責人:

香港中文大學矯形外科及創傷學系 鄭振耀教授
Department of Orthopaedics & Traumatology, CUHK Prof. JCY Cheng

Appendix IV

香港中文大學矯形外科及創傷學系
Department of Orthopaedics & Traumatology
The Chinese University of Hong Kong

青少年特發性脊柱側彎研究計劃
Adolescence Idiopathic Scoliosis Research Study

參加者同意書
Consent Form

下述簽名者 (Participant Detail)

姓名(Name) _____ 身份證號碼 (HKID No.) _____ 編號 (Code) _____

我在此聲明自願參加青少年特發性脊柱側彎研究計劃：
I declare that I consent voluntarily to take part in the Adolescence Idiopathic Scoliosis Research Study:

1. 我已經完全瞭解及自願參予此計劃。
   I fully understand and voluntarily join the research study.
2. 我明白此計劃是怎樣進行的。
   I fully understand the details of the study including its aims, methods and use of data.
3. 我有權提出有關此計劃的各種問題，而且我所提出的問題會得到完滿的解答。
   I have the right to ask any question about the study and have satisfactory answer for the researchers/doctors.
4. 我明白有權隨時退出此計劃，我亦明白醫護人員有權決定我不適宜完成此計劃而須要停止，並不會影響正常的醫療照顧。
   I understand that I have the right to withdraw from the study and the healthcare personnel/researchers also have the right to ask my withdrawal due to inappropriate continuation of the study without affecting normal medical care.
5. 我提供的資料準確無誤，並有可能用作醫學研究用途。
   All the information provided by me is correct and will be used for research purposes.
6. 我證明我已經完全瞭解這份同意書的內容。
   I acknowledge that I understand the contents of this consent form.
7. 如有任何問題，可於辦工時間內致電 2632-3309 向楊先生查詢。
   If you have any question, please contact Mr. Yeung at 2632-2745 during office hour.

參加者 (Participant)	家長/監護人 (Parent / Guardian)
簽名(Signature)	簽名(Signature)
姓名(Name)	姓名(Name)

茲證明上述自願者完全了解他們所參加的計劃，並表示同意書正確無誤。

工作人員姓名：_____ 日期：_____
(Researcher Name) (Date)

Appendix V

香港中文大學
矯形外科及創傷學系

青少年特發性脊柱側彎研究計劃
生物化學測試及活組織檢查
參加者同意書

姓名: _____ 性別: _____

身份証/護照號碼*: _____ 出生日期: 年 月 日

我因特發性脊柱側彎，須接受脊椎融合手術。手術日期為主診醫生安排。經由醫生解釋所涉及研究是有關骨質生長及活組織檢查，我已明白這次研究的目的是為了解特發性脊柱側彎的起因。

我 同意／不同意* 醫生在手術過程中取出:

- 盤骨 / 髂骨中一片 0.5 立方毫米的小骨片 *。
- 0.5 平方毫米皮膚 *

經解釋後，我明白到取小骨片及皮膚對手術沒有影響，亦不會產生其他的併發症。

病人	父母 / 監護人*	
姓名	姓名	
簽名	簽名	日期(日/月/年)

研究人員	
姓名	
簽名	日期(日/月/年)

*請刪除不適用者

Appendix VI

Department of Orthopaedics & Traumatology
The Chinese University of Hong Kong

Adolescent Idiopathic Scoliosis Research Study

Consent form for Bone Metabolism Study and Bone Biopsy

Patient Name: _____ Sex: _____

HKID/Passport No.*: _____ Date of Birth: D M Y

I am affected by idiopathic scoliosis and will undergo posterior spinal fusion with implantation and autologous bone graft on the designated date. A study of bone growth in adolescent idiopathic scoliosis will be carried out. An antibiotic will be given by the doctor before the surgery. Hereby I consent / do not consent* that I take tetracycline according to the doctor instruction. I consent / do not consent* during the surgery a block of 0.5cm^3 bone biopsy from the bone graft site will be used for research in Idiopathic Scoliosis. While read the patient information sheet, I understand that Dr. _____ (Name of Doctor) had explained to me that the possible effects of tetracycline and the bone specimen taken for examination would not affect the result of the operation, nor does it add any further problem to harvest of bone graft. It would not cause any extra complications.

Patient	Parent / Guardian*	
Name in block	Name in block	
Signature	Signature	Date (dd/mm/yy)

Medical Practitioner	
Name in block	
Signature	Date (dd/mm/yy)

* Please delete if inapplicable